Response of dendritic cells to *Mycobacterium tuberculosis* infection and the induction of protective immunity using dendritic cells infected with an auxotrophic mutant of *M. tuberculosis*.

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Abstract

Mycobacterium tuberculosis, the aetiological agent of tuberculosis, is an intracellular pathogen commonly infecting macrophages, and has also been shown to infect dendritic cells (DC). As DC are particularly effective antigen-presenting cells, it is likely that they play a principle role in initiating anti-mycobacterial T cell responses. This work investigates the activation of DC in response to *M. tuberculosis* infection using murine bone marrow-derived DCs, generated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). It was found that both unsorted DC populations and those sorted on $CD11c^+$, were capable of supporting the survival and replication of wild type *M. tuberculosis* (H37Rv) in a manner similar to that observed in macrophages. Mycobacterial infection was found to be sufficient to activate the DC populations, particularly $CD11c^+$ DC, to acquire a mature phenotype, as measured by cytokine production and expression of costimulatory and antigen presentation molecules on the cell surface.

Further study showed that mycobacteria-infected DC could prime protective immunity in an experimental model of murine tuberculosis. This was carried out using a lysine auxotroph of *M. tuberculosis*. Infected DC were used to vaccinate syngeneic or allogeneic mice. Protection against challenge with wild type *M. tuberculosis* was observed in both cases, suggesting that recipient antigen-presenting cells cross-presented mycobacterial antigen from donor DC to induce a protective immune response. A similar protective response was observed on using a xenogeneic model, in which infected murine DC were used to vaccinate guinea pigs. Both $CD4^+$ and $CD8^+$ T cells harvested from spleens of vaccinated mice, showed specific production of interferon-γ in response to mycobacterial antigen, indicating that cross-presentation by recipient antigen-presenting cells results in the effective priming of mycobacteria-specific T cells *in vivo*.

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List of abbreviations

Ag	antigen
AIM-V	serum-free lymphocyte medium
APC	antigen-presenting cell
ATP	adenosine triphosphate
β2m	β2-microglobulin
BCG	bacille Calmette-Guerin
BMDC	bone marrow-derived dendritic cell
BMMϕ	bone marrow-derived macrophage
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CFU	colony-forming unit
ConA	concavalin A
CpG	unmethylated bacterial DNA motif
CR	complement receptor
DC	dendritic cell
DC-SIGN	dendritic cell specific ICAM-3-grabbing non-integrin
DEC-205	dendritic cell receptor for endocytosis
DN	double negative
DNA	deoxyribonucleic acid
D-NAME	nitro-D-arginine methyl ester
DOT	directly observed therapy
dsRNA	double stranded RNA
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
FSC	forward scatter characteristics
GM-CSF	granulocyte-macrophage colony stimulating factor
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IV	intravenous
LAM	lipoarabinomannan
LCMV	lymphocytic choriomeningitis virus
L-NAME	nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MACS	magnetic cell sorting
MCP	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
Mϕ	macrophage
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
NK	natural killer
NO	nitric oxide
OVA	ovalbumin
PBMC	peripheral blood mononuclear cell

PBS	phosphate buffered saline
PE	phycoerythrin
PGE2	prostaglandin E2
PPD	purified protein derivative
RANTES	regulated upon activation, normal T cell expressed, and presumably secreted
rGM-CSF	recombinant GM-CSF
RNA	ribonucleic acid
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error of mean
SSC	side scatter characteristics
TACO	tryptophan aspartate rich coat protein
TAP	transporter associated with antigen processing
TB	tuberculosis
TCR	T cell receptor
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
tsDC	temperature-sensitive dendritic cell line

Chapter 1

Introduction

1.1 *Mycobacterium tuberculosis*

The genus Mycobacterium consists of non-motile, non-spore-forming aerobic bacilli approximately 1μm in length. They possess a cell wall rich in lipids, making their surface hydrophobic and conferring resistance to many disinfectants. This cell wall also conveys their characteristic staining properties, once stained the bacteria cannot be decolourized with acid solutions, hence the name acid-fast bacilli. *Mycobacterium tuberculosis* belongs to the slow-growing family of mycobacteria, dividing approximately once every 20 hours. This slow growth forms the basis of the chronic nature of infection, complicates microbiological diagnosis and necessitates long-term drug treatment. *M. tuberculosis* is a facultative intracellular bacterium, as it has the capacity to survive and replicate within phagocytes, adapting to enable survival within the hostile environment of the host.

1.1.1 Mycobacterial cell wall

Mycobacteria have a complex, lipid-rich cell wall, which is responsible for many of their characteristic properties: acid-fastness, slow-growth, resistance to detergents, resistance to common antibiotics and antigenicity. The cell wall skeleton contains peptidoglycan and arabinogalactan with the highly immunologically active molecule lipioarabinomannan (LAM). The cell wall also contains multiple layers of long-chain fatty acids, called mycolic acids, which form a permeability barrier to polar molecules – for review see (46).

The outer layer of the cell envelope, or capsule, consists of a variety of polysaccharides, lipids and proteins. Capsular components have been suggested to be involved in pathogenicity, for example contributing to adhesion and penetration into the host cell and protecting the bacterium within the host environment (47).

The mycobacterial cell envelope is an important target for anti-mycobacterial drugs. Several of the existing drugs target enzymes involved in cell wall biosynthesis, for example isoniazid targets mycolic acid synthesis (242) and ethambutol is thought to inhibit the incorporation of glucose into arabinomannan and arabinogalactan (218). The cell envelope is also important for the transfer of molecules in and out of mycobacteria. The high lipid content creates a permeability barrier, however pore-forming molecules, or porins, are thought to create hydrophilic channels, allowing access of polar molecules across the cell envelope (198).

The cell envelope is a dynamic structure in growing mycobacteria, with molecules moving both within and through the envelope. Even the stable cell wall skeleton is continuously being reconstructed (46).

1.1.2 *M. tuberculosis* genome

The mycobacterial genome, as for most bacteria, consists of a single circular chromosome plus extrachromosomal elements such as plasmids and phages. Mycobacteria belong to the high cytosine/guanine (C+G) group of Gram positive bacteria, with C+G content of 62-70%, with the exception of *M. leprae*, which has C+G content of 56%.

The most commonly used laboratory strain of *M. tuberculosis*, H37Rv, has now been completely sequenced (40). Approximately 4,000 genes have been identified, around 40% are of clearly defined function, 40% shown to belong to previously identified classes of genes and 20% are completely unknown.

A number of broad features have been described from this vast amount of genomic information;

- *M. tuberculosis* has a much broader range of metabolic capabilities than was previously thought, including the ability to adapt to anaerobic environments.

- A significant part of the genome encodes genes involved in lipid biosynthesis and metabolism.
- *M. tuberculosis* has a wide range of mechanisms for different levels of gene regulation, including 13 sigma factors, several eukaryotic-like serine/threonine protein kinases, 11 sensor histidine kinases and over 100 transcriptional regulator proteins.
- Around 10% of the genome encodes a family of closely related genes, known as PGRS sequences. The function of this highly conserved family is unknown, but a possible role in antigenic variation or virulence has been suggested (39).

A current challenge is to exploit this genomic information in order to gain an understanding of the molecular basis of mycobacterial pathogenicity, immunogenicity and immunopathology. A range of post-genomic techniques are available to facilitate this type of analysis. Comparative genomics involves the detailed comparison of the genomes of closely related strains or species. In addition to the H37Rv genome, a variety of other mycobacterial genomes are currently being sequenced, including BCG, *M. leprae*, *M. bovis* and *M. smegmatis*. Comparisons of these genomes may provide important insights into the molecular basis of pathogenicity and host range.

Targeted gene deletion techniques or random mutagenesis approaches (using transposons, illegitimate recombination or signature-tagged mutagenesis) are available to identify genes of unknown function or those which may be important in pathogenesis (39). Techniques such as proteomics (114) and microarray analysis (11) may be used to investigate the global genomic response to different growth conditions. Such techniques shall be used over the next few years to understand many of the genes important for intracellular growth and survival of the bacteria.

1.1.3 *M. tuberculosis* as a pathogen of man

In 1882, Robert Koch first identified *M. tuberculosis* as the etiological agent of tuberculosis (TB), and today it remains one of the most successful pathogens of mankind with around one third of the world population infected and causing more than 2 million deaths per year (239). The incidence of tuberculosis has increased in recent years and this can be attributed to many factors including co-infection with human immunodeficiency virus (HIV), breakdown of social and healthcare infrastructures in many parts of the world, increased migration of people and the emergence of multi-drug resistant strains (145).

Tuberculosis is transmitted though the air via droplets which are inhaled and reach the alveoli. Pulmonary TB begins with infection in the alveolar spaces of the lungs. *M. tuberculosis* is phagocytosed by resident alveolar macrophages (Mϕ) and additional phagocytes are attracted to the site of infection by inflammatory cytokines and chemokines. Accumulation of cells at the focus of infection generates a granuloma, or 'tubercle'. *M. tuberculosis* is able to survive within the hostile environment of granuloma Mϕ and latent viable bacteria may persist in the granuloma for many years. This ability to survive within the Mϕ is what clearly defines *M. tuberculosis* as a human pathogen.

Once infected, most individuals mount an adequate immune response and do not develop clinical disease, the infection may remain latent for the duration of the individuals lifetime. However, latent infection can develop into clinical TB, usually as a consequence of some form of immunodeficiency, such as co-infection with HIV, corticosteroid therapy, ageing, nutritional factors or drug/alcohol abuse. This results in clinical disease known as reactivation, or secondary TB. Primary TB, where clinical symptoms develop shortly after infection, is also most likely in individuals whose immune system is impaired.

Clinical TB is treated with a combination of antibiotics, including rifampicin, isoniazid, ethambutol, streptomycin and pyrazinamide. A complete course of treatment lasts approximately 6 months and if the individual is infected with a drug-sensitive strain and the course of treatment is adhered to, this is usually successful. Poor compliance with the treatment regimen or inappropriate treatment increases the risk of relapse and favours the development of secondary drug resistance. In order to combat the problem of non-compliance the World Health Organization advocates DOTS (directly observed treatment, short course) for every TB patient, where administration of every dose is supervised by a healthcare or social worker. In most areas where DOT has been implemented it has given significant improvements in cure rates. However, DOTS regimens are very labour intensive and are challenging to deliver in many developing countries, thus only 1 in 5 patients receives DOTS worldwide (239).

Approximately 90% infections remain latent and sub-clinical, thus the development of novel strategies which can eliminate latent organisms is a major challenge for the future control of TB.

1.2 The immune response to *M. tuberculosis*

The main reason for the success of *M. tuberculosis* as a human pathogen is its ability to persist in an immunocompetent host. The host immune response to TB involves a complex series of interactions of different cell populations in order to control and contain the infection. Following infection with *M. tuberculosis* there are four potential outcomes:

- Initial host response is completely effective and all bacteria are eradicated.
- Mycobacteria grow and multiply, causing clinical disease (primary TB).

- Bacilli become dormant, giving latent infection.
- Latent bacilli begin to replicate, resulting in clinical disease (reactivation TB).

Less than 10% of infections lead to primary TB, in most infected individuals the immune response is sufficient to control infection, however the risk of reactivation in later life remains.

The immune response to *M. tuberculosis* can be divided into three stages. During the early (innate) response the bacteria are taken up by host phagocytes, which are then activated to induce an inflammatory response. This is followed by the late (acquired) response in which specific T cells are activated, resulting in a cell-mediated response. In chronic/persistent infection mycobacteria are contained within a granuloma, consisting of Mϕ, T cells and multi-nucleated giant cells. Bacteria within the granuloma may be;

- in a dormant, non-replicating state
- replicating, but killed by the immune system
- metabolically altered, with limited/infrequent replication cycles

The granuloma often has central necrosis, which may serve to eliminate infected cells and provide an anoxic environment in which the bacilli cannot survive, thus tissue injury may serve a protective function. Caseating granulomas and the associated fibrosis are the main cause of tissue pathology in TB.

1.2.1 Mycobacterial interaction with Mϕ

M. tuberculosis is transmitted via the respiratory route. Droplets containing small numbers of bacteria (1-3 bacilli are sufficient to establish infection) are inhaled into the lung and here the bacteria are taken up by alveolar Mϕ. Invasion of the host cell is not necessarily a destructive process as the pathogen relies on the host cell for

nutrients. Infected alveolar Mϕ adhere to and spread over the alveolar surface, allowing erosion of the bacteria into the interstitium. This causes local tissue damage and release of inflammatory mediators which recruit Mϕ, thus facilitating the spread of infection. The host Mϕ respond to infection by producing a variety of toxic effectors in order to eliminate the pathogen. Thus, a dynamic balance is established between the bacterium and the host response.

1.2.1.1 *M. tuberculosis* entry into the Mϕ

A number of Mϕ receptors have been identified which can interact with *M. tuberculosis* and facilitate its uptake, including complement receptors, Fc receptors, Mϕ mannose receptor and CD14, reviewed in (60). The choice of receptor used for entry to the Mϕ may influence the subsequent response to infection. For example, uptake of IgG-opsonized *M. tuberculosis* via FcγR has been shown to induce the production of toxic oxygen radicals and permit phagolysosome fusion (5). In contrast, uptake via complement receptor CR3 prevents activation of the respiratory burst and arrests phagosome maturation (133). Cholesterol has been shown to be essential for entry of mycobacteria into the Mϕ, as depletion of cholesterol from the plasma membrane resulted in inhibition of uptake (77). Over the last few years much research has focused on the interaction of mycobacteria with Toll-like receptors (TLRs). This family of receptors was originally identified in *Drosophila* and was found to be important in resistance to microbial pathogens. A number of Toll homologues have since been identified in mammals. TLR2 and TLR4 have both been implicated in the activation of Mϕ by mycobacteria (179). Thus, a number of interactions of *M. tuberculosis* with Mϕ receptors can initiate intracellular signalling within the host cell, and so direct the subsequent host response.

1.2.1.2 Anti-mycobacterial functions of the Mϕ

The prime function of Mϕ is the eradication of engulfed microbes, and therefore uptake of *M. tuberculosis* stimulates Mϕ activation and initiation of effector mechanisms which attempt to destroy the pathogen.

Once phagocytosed by the Mϕ, *M. tuberculosis* resides within the phagosome. This vacuolar compartment fuses with lysosomes; this is a complex event involving the interaction of the phagosome with various endocytic vacuoles and results in the formation of a phagolysosome. Thus, the bacteria contained within the phagosome are exposed to degradative lysosomal enzymes.

The most studied anti-mycobacterial mechanism of activated Mϕ is the inducible nitric oxide synthase (iNOS) dependent pathway, which generates toxic nitrogen intermediates (RNI) such as nitric oxide (NO). RNI are generated during the conversion of L-arginine to citrullene by iNOS, and are important mycobactericidal effector molecules. Murine Mϕ have previously been shown to produce RNI in response to *M. tuberculosis* infection, and that these RNI induced mycobacterial killing (32). Mice deficient in iNOS or in which the RNI pathway has been inhibited have been reported to be extremely susceptible to mycobacterial infection (146). iNOS is expressed in the granuloma throughout the persistent phase of infection in mice, and inhibition of RNI production results in the reactivation of persistent infection (71). Thus, although iNOS is essential for the control of both acute and chronic infections, RNI fail to eradicate the bacterium and so may promote persistence.

Oxygen dependent mechanisms of mycobacterial killing involve the formation of reactive oxygen intermediates (ROI) such as hydrogen peroxide (H_2O_2) or the superoxide anion. These ROI can cause lipid peroxidation, cell membrane damage, and can also damage DNA and proteins. There is however some controversy over whether ROI contribute to mycobacterial killing, one report (32) has suggested that

such mechanisms are unlikely to be significantly involved. Also it has been shown that NADPH oxidase-deficient mice (unable to produce superoxide) show increased susceptibility to infection (44).

Infection of Mϕ with *M. tuberculosis* can induce programmed cell death, or apoptosis (184). The role of apoptosis in controlling the growth or killing of *M. tuberculosis* is unclear. It has been suggested that apoptosis of cells infected with intracellular pathogens may be beneficial to the host, by eliminating a supportive environment for bacterial growth (119), or by preventing the spread of the pathogen by sequestration in apoptotic bodies (73). It is likely that the mechanism of inducing apoptosis is important in determining the outcome for intracellular mycobacteria. One group have compared the fate of intracellular BCG when Mϕ were induced to apoptose via complement-mediated lysis, Fas ligation, CD69 ligation and addition of exogenous ATP (128). Only ATP was found to induce both cell death and killing of BCG. In contrast, others reported that Fas ligation caused apoptosis reducing the viability of intracellular mycobacteria (161).

Thus, a variety of mechanisms are utilized by Mϕ in an attempt to control the growth of *M. tuberculosis*. It is likely that these mechanisms act synergistically to control the intracellular growth of the bacterium.

1.2.1.3 *M. tuberculosis* evasion of microbicidal mechanisms

M. tuberculosis has the ability to persist within the Mϕ despite the activation of effector mechanisms to eliminate the pathogen. This is largely due to the fact that the bacterium has evolved a number of strategies to combat the destructive effects of the Mϕ environment.

In order to persist within the phagosome, *M. tuberculosis* prevents the fusion of the phagosome with lysosomes, thus preventing formation of the phagolysosome and

exposure to degradative lysosomal enzymes. This was first observed by (6), and similar mechanisms have been reported to be utilized by *Salmonella*, *Legionella* and *Bordetella* (88, 106, 207). *M. tuberculosis* arrests endosomal trafficking at an early stage, thus preventing maturation of the phagosome. The most important characteristics of the mycobacterial phagosome are incomplete luminal acidification (215) and the absence of mature lysosomal hydrolases (141). Despite the block in acquisition of lysosomal compartments, the mycobacterial phagosome is not a static organelle, as demonstrated by access to transferring-bound iron (214) and the accumulation of transferring receptors (36). Two organellar systems contribute to phagosomal maturation; the interaction and exchange of material with early and late endosomes (35) and the delivery of membrane and luminal cargo from the trans-Golgi network to the phagosome (75).

The block in maturation of the mycobacterial phagosome occurs between the small GTP-binding proteins, Rab5 (early endocytic) and Rab7 (late endosomal), which direct membrane trafficking in eukaryotic cells. Rab5 is found to accumulate on mycobacterial phagosomes, whereas Rab7 is not detected at the usual times for its recruitment (235). Early endosomal autoantigen 1 (EEA-1) is a Rab 5 effector which functions as an organelle tethering molecule by bridging membranes for fusion (33). EEA-1 directly interacts with Rab5 and also binds PI3P (phosphatidylinositol 3-phosphate) (204). PI3P is generated on the endosomal membrane by the action of another Rab5 effector PI3K (phosphatidylinositol 3-kinase) (34). Thus, the reduced or altered recruitment of EEA-1 has led to the investigation of PI3P and PI3K in the maturation block.

Stress-induced p38 MAPK (mitogen-activated protein kinase) has been implicated as a component of *M. tuberculosis* phagosome maturation arrest, linked to reduced EEA-1 recruitment (74). Mycobacterial uptake stimulates p38 phosphorylation. Inhibition of p38 MAPK increases EEA-1 co localization with mycobacterial

phagosomes, leading to phagosomal acidification and the accumulation of late endocytic markers. *M. tuberculosis* specifically inhibits a sphingosine kinase-dependent Ca^{2+} rise which affects phagosome maturation (142). EEA-1 is recruited to membranes by Rab5 and PI3P, the product of PI3K. Ca^{2+} and calmodulin influence the recruitment of PI3K to phagosomal membranes in the macrophage (233). Thus, linking mycobacteria-dependent inhibition of Ca^{2+} rise with anomalous recruitment of PI3P-dependent tethering molecules to mycobacterial phagosomes. *M. tuberculosis* analogs of mammalian phosphatidylinositols have been studied in the context of the phagosomal maturation block. PIM (phosphatidylinositol mannoside) and LAM (lipoarabinomannan) have been shown to intercalate into endomembranes and traffic within mycobacteria-infected cells (9). LAM has been demonstrated to inhibit phagosomal maturation and block a PI3K-dependent pathway of trafficking between the trans-Golgi network and the phagosome, which is essential for maturation (75). PIM is an *M. tuberculosis* mimic of mammalian phosphatidylinositols and it specifically stimulates fusion of early endosomes, this is dependent on Rabs, but is PI3k-independent (234). Thus, PIM maintains the early endosomal nature of the phagosome. Thus, *M. tuberculosis* lipids can modulate the host cell membrane sorting and organelle biogenesis processes; LAM blocks trafficking of lysosomal constituents from the trans-Golgi network to the phagosome (e.g. H^+ ATPase components and lysosomal hydrolases) and PIM stimulates fusion between *M. tuberculosis* phagosomes and early endosomes.

Mycobacteria can also influence phagosome maturation via interaction with TACO (tryptophan aspartate rich coat proteins). As previously mentioned, cholesterol is required for uptake of *M. tuberculosis*, the bacterium is phagocytosed at cholesterol-rich domains of the Mϕ plasma membrane (77); this ensures that the phagosome is coated with TACO. Retention of TACO on the surface of the mycobacteria-containing phagosome prevents phagolysosome fusion (63). *M. tuberculosis* can

also maintain the intraphagosomal environment to promote its survival. The exclusion of the vesicular H^+ ATPase from *M. tuberculosis* phagosomes (244), prevents their acidification and thus the bacteria avoid the harmful effects of an acidic environment. Thus, it would appear that *M. tuberculosis* can avoid the hostile environment of the phagosome via a number of mechanisms.

Mycobacteria also have a number of defence mechanisms to counteract the toxic effects of ROI and RNI. These toxic molecules have been shown to damage microbial DNA, proteins and lipids. Due to the diversity of oxidative and nitrosative stresses it is likely that *M. tuberculosis* has evolved a variety of resistance mechanisms, including;

- Avoidance or subversion of host phagocytes – for example the use of complement receptors CR1 and CR3 which have been shown not to trigger the oxidative burst (194, 243).
- Production of anti-oxidant enzymes – production of superoxide dismutase and catalase, which are capable of degrading ROI (108).
- Production of molecular scavengers – such as cell wall LAM, which is a scavenger of oxygen radicals (31).

1.2.1.4 Role of the Mϕ in initiating the immune response

As shown above, the Mϕ is the host cell in which *M. tuberculosis* can persist, or be killed as a result of anti-mycobacterial mechanisms. In addition to these anti-microbial effector functions, the Mϕ plays a role, along with dendritic cells, in initiating both innate and acquired immune responses. On infection with *M. tuberculosis* Mϕ are stimulated to produce a variety of inflammatory mediators. Pro-inflammatory cytokines IL-1β (240), TNF-α (185) and IL-12 (123) have been shown to be up-regulated in *M. tuberculosis*-infected Mϕ. A wide range of chemokines

have also been reported to be produced by Mϕ in response to mycobacterial infection, including IL-8, osteopontin, MCP-1, MIP-1α and RANTES (176, 187). Thus, it seems that a crucial role of Mϕ following infection is to mobilize and recruit a wide range of other immunomodulatory and effector cells to the site of infection.
In addition to release of cytokines and chemokines, Mϕ can also initiate the cellular response, as they can function as antigen-presenting cells (APC) (230). Dendritic cells are the most efficient APC (see section 1.4), however Mϕ are also capable of processing and presenting antigens in association with MHC class I and II molecules to T cells. Previous studies have shown that mycobacteria-infected Mϕ have a reduced capacity to present antigen, especially in the context of MHC class II (168), either by inhibiting the expression of MHC class II molecules or by inhibiting antigen processing (159, 160). *M. tuberculosis* may, therefore, evade recognition by specific T cells by reducing the antigen presenting capacity of the cell in which it resides.

1.2.2 The cytokine network

The modulation of the immune response to all pathogens is dependent on the production of cytokines. These molecules are responsible for cell recruitment and the differentiation of naïve T cells into effector cells. IL-12 promotes the development of naïve T cells into a Th1 phenotype, which produce principally IFN-γ; whereas IL-4 promotes the development of Th2 cells which produce IL-4 and IL-10 (162). It is generally believed that resolution of mycobacterial infection requires a primarily Th1-type response.
M. tuberculosis strongly induces a variety of cytokines during infection. The inflammatory response to the pathogen is crucial to the control of infection, but may also contribute to the associated pathology and clinical manifestations, for example

IL-1β and TNF-α are associated with producing fever and wasting. A selection of cytokines involved in the host response to mycobacterial infection are discussed below, however this is by no means exhaustive.

1.2.2.1 Interleukin-12

As stated above, the control of *M. tuberculosis* infection is based upon the development of a Th1 type response. IL-12 is produced by both Mϕ and dendritic cells in response to mycobacterial infection (93, 123), which in turn drives the development of a Th1 response with production of IFN-γ. IL-12 is an essential cytokine is the control of *M. tuberculosis* infection. The administration of exogenous IL-12 to mice during early infection resulted in significantly reduced bacterial burden and increased survival time, although ultimately the mice still succumbed to infection (70). This increased resistance to infection was not due to a shift from Th2 to Th1 response, as no measurable Th2 response was observed in infected mice. Compelling evidence for the importance of IL-12 in control of mycobacterial infection was provided by the use of IL-12p40 gene-deficient mice. These mice showed increased bacterial loads and decreased survival time as compared to wild type mice, probably due to diminished IFN-γ production (43). Humans with mutations in IL-12 or IL-12 receptor genes are more susceptible to disseminated mycobacterial infections (166). Administration of IL-12 DNA has been shown to be capable of substantially reducing bacterial numbers on the lungs of chronically infected mice (138), suggesting that induction of this cytokine could be important when considering future vaccine design.

1.2.2.2 Interferon-γ

IFN-γ is a key player in control of *M. tuberculosis* infection. This important cytokine is produced by $CD4^+$ and $CD8^+$ T cells, as well as NK cells in response to

mycobacterial infection. IFN-γ knockout mice are extremely susceptible to *M. tuberculosis* infection (41, 67). The organs of these mice showed increased bacterial growth and although granulomas were formed they quickly became necrotic. These mice showed defective Mϕ activation and low expression of iNOS, which is the most likely cause of increased susceptibility. The survival time of $iNOS^{-/-}$ mice was almost double that observed in IFN-γ knockout mice (67, 146), suggesting that there may be IFN-γ-dependent iNOS-independent mechanisms of protection. The importance of IFN-γ can also be observed in humans with defective genes for IFN-γ or IFN-γ receptor, as these individuals are prone to suffer from serious mycobacterial infections (166).

However, increased levels of this cytokine may not be a reliable correlate of protection, as it has been shown that *M. tuberculosis* can diminish the ability of Mϕ to respond to IFN-γ (223). Thus, the levels of IFN-γ produced in response to infection may be less predictive of outcome of infection than the ability of the Mϕ to respond and initiate anti-mycobacterial functions.

1.2.2.3 Interleukin-4

There is much controversy surrounding the presence of Th2 responses in tuberculosis. *M. tuberculosis* is a potent inducer of IL-12, thus Th1 responses are almost invariably found. However, IL-4 detection is variable, and although there have been reports of Th2 responses in TB, they can be difficult to demonstrate. In humans, it has been shown that the Th1 response is depressed, but this does not necessarily correlate with an enhanced Th2 response (247). A similar situation can be shown in the mouse model of experimental TB infection. BALB/c mice, which are more susceptible than C57Bl/6 mice to infection, do not show a polarized Th2 response, although resistance can be increased by administration of exogenous IL-

12 (70). IL-4-deficient mice were shown to be equally resistant to infection as were control mice (158). These reports would indicate that the absence of a Th1 response does not necessarily induce a Th2 response.

1.2.2.4 Interleukin-10

IL-10 is an anti-inflammatory cytokine produced by Mϕ and T cells during *M. tuberculosis* infection. It is thought to deactivate Mϕ, down-regulate IL-12 and thus, in turn decrease IFN-γ production. IL-10 acts to directly inhibit $CD4^+$ T cells responses and also to inhibit the APC function of infected cells (185). Transgenic mice over-expressing IL-10 were less efficient at clearing mycobacterial infection than control mice, however T cell responses were unaffected (154). IL-10 was seen not to be responsible for a failure of Th1 response, as IL-10 knockout mice were no more capable of clearing infection that were wild type mice (113). In another model of transgenic mice over-expressing IL-10, it was demonstrated that mice over-producing this cytokine were more prone to reactivation of persistent infection (226). Thus, IL-10 may play an important role during the chronic stage of infection, promoting reactivation.

1.2.2.5 Tumor necrosis factor-α

TNF-α plays many important roles in both immunity and pathology of TB. *M. tuberculosis* induces production of TNF-α by Mϕ, dendritic cells and T cells (93, 123). TNF-α is essential for the control of acute infection, as TNF-α-deficient mice showed increased susceptibility to *M. tuberculosis* infection, with increased bacterial loads in comparison to control mice (68). This is probably due to its role in Mϕ activation, as TNF-α works in synergy with IFN-γ to activate Mϕ to carry out anti-

mycobacterial effector functions (32). Also, in the absence of TNFα the granulomatous response is impaired (68), the granulomas that form are disorganized with fewer activated Mϕ. Thus, TNF-α also affects cell migration to the tissues in *M. tuberculosis* infection, probably through influencing expression of adhesion molecules and/or chemokines. TNF-α has also been demonstrated to be responsible for much of the resultant immunopathology observed in TB (116), emphasizing the balance between protective immunity and immunopathology.

1.2.2.6 Other cytokines implicated in response to mycobacterial infection

IL-6 has various roles in the immune response including haematopoeisis, inflammation and T cell differentiation, and it has been implicated as being involved in the host response to TB. This cytokine has been demonstrated to be produced by Mϕ in response to infection with mycobacteria and a potential role in suppression of T cell responses has been suggested (232).

Transforming growth factor-β (TGF-β) is an anti-inflammatory cytokine which has been reported to inhibit T cell responses to *M. tuberculosis* (186). However, the role of TGF-β in protection or pathology of TB is not well understood.

IL-15 has been shown to play a role in mycobacterial infection, principally via increasing NK and cytotoxic T cell responses (229). IL-18 has also been implicated and is thought to induce the production of IFN-γ in a manner similar to IL-12, but to a lesser extent (121).

1.2.3 Cell-mediated response

The protective response to *M. tuberculosis* is dependent on cell-mediated immunity; as the pathogen is intracellular, T cell effector mechanisms rather than antibodies

are required to control or eliminate infection. In the past, research has focused on the $CD4^+$ T cell response, however in recent years $CD8^+$ T cells and other minor T cell populations have also been shown to play a role in protection.

In the murine model of experimental TB, within 1 week of infection there are elevated number of $CD4^+$ and $CD8^+$ T cells in the lymph nodes draining the lung; by 2-4 weeks post-infection both $CD4^+$ and $CD8^+$ T cells have migrated to the lung and express an effector/memory phenotype (62, 199). This demonstrates that activated T cells travel to the infection site and interact with APCs. The granuloma contains both $CD4^+$ and $CD8^+$ T cells (177) and it is likely that these are involved in containing the infection and preventing reactivation.

1.2.3.1 $CD4^+$ T cells

As *M. tuberculosis* is contained within vacuolar compartments within the host cell, it is not surprising that mycobacterial antigens are processed and presented on MHC class II molecules for recognition by $CD4^+$ T cells. Many studies utilizing antibody depletion (152), adoptive transfer (164, 165) and use of gene-disrupted mice (155, 221) have shown that $CD4^+$ T cells are required for the control of infection. In humans, co-infection with HIV has demonstrated that loss of $CD4^+$ T cells results in increased susceptibility to both acute and reactivation TB.

The main function of $CD4^+$ T cells is thought to be the production of IFN-γ, and possibly other cytokines, which activate Mϕ to perform antimicrobial functions and control the infection. MHC class $II^{-/-}$ and $CD4^{-/-}$ mice showed diminished levels of IFN-γ during the early phase of infection (155, 221). However, by 3 weeks post-infection levels of IFN-γ in the lungs were similar to that observed in wild type mice, with $CD8^+$ T cells contributing to IFN-γ production. Late IFN-γ production did not rescue the mice and they succumbed to infection. Expression of iNOS by Mϕ was

also delayed in CD4-deficient mice but returned to normal levels with IFN-γ production. In a murine model of chronic persistent infection (191), CD4 depletion caused rapid reactivation. Levels of IFN-γ were similar to that observed in control mice and no change in Mϕ expression of iNOS was observed, suggesting that there are IFN-γ- and iNOS-independent $CD4^+$ T cell mechanisms for the control of TB.

A possible role for $CD4^+$ T cells is in activating APC. Ligation of CD40 on Mϕ or dendritic cells by CD40L on $CD4^+$ T cells results in increased antigen presentation and costimulatory activity. This may be of importance in *M. tuberculosis* infection, however it has been demonstrated that CD40L-deficient mice were not more susceptible to acute infection (26). A more recent report shows that $CD40^{-/-}$ but not $CD40L^{-/-}$ mice succumbed to aerosol infection (132), probably due to the presence of an alternative ligand for CD40.

$CD4^+$ T cells have also been shown to exert cytolytic functions and this may play a role in controlling infection. $CD4^+$ T cells have been shown to produce perforin and granulysin (27), although this mechanism did not appear to contribute to bacterial killing. FasL- or TNF-α-induced apoptosis impact on *M. tuberculosis* viability is controversial, some report a reduction in bacterial numbers following apoptosis, while others suggest this mechanism has little effect (161, 203).

As discussed in section 1.2.1.4, infection of Mϕ with *M. tuberculosis* appears to diminish their capacity to present mycobacterial antigens in association with MHC class II to $CD4^+$ T cells, which could possibly contribute to the inability of the host to eliminate a persistent infection.

1.2.3.2 $CD8^+$ T cells

Despite the intraphagosomal location of mycobacteria, it is now widely accepted that $CD8^+$ T cells are involved in the protective response against infection. The

presence of mycobacteria-specific $CD8^+$ T cells in humans has been widely reported (126, 136, 205). However, it is work conducted in mice which has supplied compelling evidence for the involvement of these cells in protective immunity against mycobacteria. Mice deficient in β2-microglobulin (β2m) (69), transporter associated with antigen processing (TAP) (10), perforin (42) or CD8α (206) were all shown to be more susceptible to infection with *M. tuberculosis* than wild type mice, but to different extents. One group conducted a direct comparison of these mouse strains (206) and discovered that β2m-deficient mice were most susceptible, followed by TAP-deficient and CD8α-deficient mice. This suggests that MHC class I processing and activation of $CD8^+$ T cells is required for the control of infection. The observed increase in susceptibility of β2m-deficient mice may not be completely due to lack of MHC class I-restricted $CD8^+$ T cells. β2m is also involved in stabilizing non-classical non-polymorphic MHC Ib and CD1 molecules, which have also been shown to be involved in the response to mycobacteria. In addition to this, β2m interacts with a protein involved in iron absorption, HFe, as a consequence of this β2m-deficient mice suffer from tissue iron overload, which has been shown to increase susceptibility to TB (151).

In addition to classical MHC class I-restricted $CD8^+$ T cells, other $CD8^+$ T cells which are not restricted by MHC class I have been identified. A population of $CD8^+$ T cells recognize mycobacterial lipid and glycolipid antigens in the context of CD1 molecules (118, 228). Also identified are a class of $CD8^+$ T cells which recognize mycobacterial antigens presented in the context of non-classical MHC Ib molecules (136). The equivalent of MHC Ib molecules in mice are H2-M3 which present N-formylated peptides and mice immunized with H2-M3-binding N-formylated peptides showed immune reactivity against *M. tuberculosis* (58).

It would appear that there are two potential effector functions for $CD8^+$ T cells in TB; cytolysis of infected cells and production of cytokines, particularly IFN-γ. Transfer of

both $CD4^+$ and $CD8^+$ T cells into *M. tuberculosis*-infected mice provides equivalent levels of protection, however transfer of $CD8^+$ T cells from mice with a targeted deletion in the IFN-γ gene was unable to provide protection (221), suggesting that early during infection the most important role of $CD8^+$ T cells is production of IFN-γ. This is supported by findings that mice lacking cytotoxic T cell mechanisms show little difference from wild type mice in their ability to control infection (42, 129). Despite these findings, it has been shown that killing of infected Mϕ may result in a reduction in viability of intracellular mycobacteria. MHC class I-restricted $CD8^+$ T cells have been shown to kill infected Mϕ in a perforin-dependent manner (199). In addition, CD1-restricted $CD8^+$ T cells were demonstrated to kill infected Mϕ via granule exocytosis and this was associated with a reduction in the viability of intracellular *M. tuberculosis* (209). Thus, it is likely that the cytolytic function of $CD8^+$ T cells may contribute to control of infection via the granulysin/perforin pathway.

1.2.3.3 Unconventional T cells

In addition to $CD4^+$ and $CD8^+$ T cells, other minor T cell populations which recognize mycobacterial products have been identified. T cells expressing the γδ T cell receptor (TCR) have been implicated in response to mycobacterial infections, and have been shown to accumulate during early infection in experimental infection of mice (85). δ TCR gene-disrupted mice infected with *M. tuberculosis* did not show increased susceptibility to infection compared to wild type animals, but showed pyogenic granulomas with substantial neutrophil infiltration, suggesting a potential role for these cells in directing cellular trafficking and granuloma formation (59). Also, double negative (DN; $CD4^-CD8^-$) CD1-restricted T cells which recognize mycobacterial antigens and are capable of producing IFN-γ and expressing cytotoxic activity have been reported (210). In contrast to $CD8^+$ T cells, these cells exert their

cytotoxic activity via the Fas-FasL pathway, which appears to have no effect on the viability of intracellular mycobacteria.

1.2.2.4 Involvement of other cell types

In addition to T cells and antigen presenting cells, a variety of other cell types are thought to be involved in response to mycobacteria.

Natural killer (NK) cells are involved in early response to infection. They exhibit enhanced lytic ability against *M. tuberculosis*-infected monocytes following stimulation with IL-12 (54) and are thought to be an early source of IFN-γ prior to the emergence of specific T cells. Antibody depletion of NK cells in mice resulted in increased susceptibility to *M. avium* infection (90), demonstrating that they are involved in control of mycobacterial infections.

Neutrophils, during mycobacterial infection, are recruited by T cells and Mϕ, possibly representing a mechanism to cope with secondary infections (4). Neutrophil depletion completely abrogates the protection conferred by activated Mϕ induced during mycobacterial infection (134). Most important is the immunomodulatory activity of these cells, as they have been shown to have the ability to produce TNF-α, IL-12 and IL-1β (172), hence contributing to the global response to infection.

B cells were initially considered to play no role in control of *M. tuberculosis* infection. However, some studies indicate that B cell-deficient mice, which were unable to produce antibodies of any class, were more susceptible to mycobacterial infection (237). It was subsequently shown that mice deficient in B cell production demonstrate severe lesion formation and delayed bacterial dissemination which was found to be dependent on B cells, but not antibody-dependent (19). The role of antibodies in mycobacterial infection is poorly understood, but they may have some

contribution to host response. Administration of LAM to mice was shown to cause an increase in IgM levels and could modify the course of infection (82).

1.3 Vaccination against TB

The current vaccine Bacille Calmette-Guerin (BCG) is an attenuated form of *M. bovis* and was developed in the first decade of the last century. BCG has been in use for many years and has been administered to more individuals than any other vaccine. The protective efficacy of BCG is a matter of controversy; it appears to protect against childhood manifestations of disease (military or meningeal TB) (182) but fails to protect against the most common pulmonary form of disease in adults. The levels of protection afforded by BCG are extremely variable, ranging from 0% in Malawi to 80% in the UK - reviewed in (66). There have been many hypotheses suggested to explain this observed variation and potential strategies for improvement, some of which are considered below.

It has been suggested that BCG lacks important antigens, and indeed comparative genomics has shown the absence of a number of genes from BCG compared to virulent *M. tuberculosis* (11). These deleted regions are likely to be associated with virulence and may play a role in the failure of BCG. The best characterised of these deleted regions is RD1, which is absent in all BCG sub strains and most environmental mycobacteria, but is present in all strains of the *M. tuberculosis* complex (139). Reintroducing selected genes from these deleted regions may enhance the protective efficacy of BCG. Recombinant BCG expressing the immunodominant Ag85B of *M. tuberculosis* promoted greater levels of protection that normal BCG (100). Also possible is the creation of live attenuated

mycobacterial vaccines which include all the relevant *M. tuberculosis* antigens, by knocking out genes from *M. tuberculosis*. The absence of important T cell antigens from BCG has been supported by the discovery of ESAT-6, which is encoded in the RD1 region and is the strongest T cell target isolated from *M. tuberculosis* to date (3, 23, 178). Vaccination with ESAT-6 in adjuvant (22) or encoded in DNA vaccines (115, 137) have been shown to evoke protective responses. Thus it may be possible to give improved protection utilizing vaccines based on one, or a few, *M. tuberculosis*-specific antigens.

It has been proposed that the high prevalence of environmental mycobacteria in tropical regions is the single most important factor for the low efficacy of BCG vaccination in these regions (66). In the mid-1960s it was found that exposure to environmental mycobacteria provided varying levels of protection to TB infection, thus masking protection of a later BCG vaccination (167). Another group presented an alternate possibility, that exposure to environmental mycobacteria promoted a detrimental humoral immune response which could not be overcome by BCG vaccination (94). Recently, it has been demonstrated that sensitization with environmental mycobacteria (from soil samples in Malawi – where BCG is non-protective) caused an inhibition of initial BCG replication and so only transient immune responses were induced following BCG vaccination (21). However, this resistance promoted by environmental mycobacteria was insufficient to control challenge infection with virulent *M. tuberculosis*. This is in agreement with reports that individuals in Malawi with a high degree of prior sensitization with environmental mycobacteria show much reduced IFN-γ responses following BCG vaccination (14), while the same BCG strain gave a significant response in a non-sensitized UK population (15). A potential approach to combat this issue would be the use of subunit vaccines, as it has been demonstrated in this pre-sensitization model that the protective efficacy of 2 different subunit vaccines was unaffected by prior

exposure to mycobacteria (21). Thus subunit vaccines do not appear to be influenced by prior sensitization and stimulate a T cell response, while BCG depends on initial replication for its activity.

As previously noted, BCG promotes high levels of protection against childhood manifestations of TB, however incidence of disease increases with time since vaccination (211). This waning protection correlates with increased incidence of disease in adolescence, which cannot be prevented by a BCG revaccination strategy (86). A large proportion of the population are already BCG vaccinated, thus an advantageous strategy would be to boost this existing immunity. A subunit vaccine based on Ag85A has been reported to be a successful booster of immunity induced by BCG vaccination (24).

The development of a new vaccine with improved efficacy is a priority and many approaches to achieve this goal are under investigation. As T cells are central to the protective response against TB, vaccine design should focus on priming effective T cell responses. Table 1.1 summarizes some of the potential vaccine candidates under current investigation.

Vaccine candidate	Advantages	Disadvantages	Example
Subunit vaccines			
Ag in adjuvant	Mild side effects	Restricted number T cell clones; immunogenicity depends on adjuvant	Culture filtrate (101) ESAT-6 (22)
Naked DNA	CD4 and CD8 T cells	Restricted number T cell clones; safety concerns	Hsp60 (219)
Recombinant carrier expressing Ag	CD4 and CD8 T cells	Restricted number T cell clones; safety concerns	rVaccinia expressing Ag85 (147)
Viable mycobacterial vaccines			
M. tuberculosis deletion mutant	CD4 and CD8 T cells	Safety concerns; immunosuppressive	Erp-*M.tuberculosis* (12)
Auxotrophic mutants	Improved safety	Reduced immunogenicity	Pantothenate (189) Purines (109)
Recombinant BCG expressing cytokines	Improved immunogenicity	Primarily CD4; no TB-specific Ag; safety concerns	rBCG-IL-2, IFN-γ (153)
Recombinant BCG over-expressing Ag	Improved immunogenicity	Primarily CD4; safety concerns	rBCG-30kDa secretory protein (100)
Combination vaccines			
Prime-boosts	Improved immunogenicity	Safety concerns	BCG-rVaccinia(Ag85) (84) DNA-rVaccinia(Ag85) (147)

Table 1.1 Prospective vaccine candidates under investigation.

1.4 Dendritic cells

Dendritic cells (DC) are the most efficient APC, as they have the unique capacity to induce primary immune responses. DC have characteristic stellate morphology with cytoplasmic processes and exhibit rapid motility. They are extremely potent APC, as a small number of DC can initiate strong T cell responses. DC progenitors in bone marrow give rise to circulating precursors, which home to peripheral tissues where they reside as immature DC with high antigen capture ability. Following tissue damage or microbial invasion, immature DC become activated and gain increased T cell stimulatory capacity, they migrate to lymphoid organs where they can interact with antigen-specific T cells. Thus, DC can be said to be the 'sentinels' of the immune system, alerting the acquired immune response to the presence of invaders in peripheral tissues.

1.4.1 DC subsets

In both rodents and humans various distinct DC subsets have been identified which may represent different lineages or developmental pathways. DC originate from haematopoietic precursor cells and there are two opposing models as to how the different DC subsets may arise. The functional plasticity model suggests that DC subsets represent various activation states of a single lineage, the different functional subtypes arising in response to local environmental signals. In contrast, the specialized lineage model suggests that DC subsets are a result of different developmental lineages, with precursor populations already functionally committed (201). In reality a mixture of these two models appears to exist and much controversy remains surrounding the emergence of distinct functional DC subsets.

In the presence of GM-CSF, murine bone marrow cultures yield myeloid DC ($CD11c^+CD11b^+B220^-$), however in the presence of Flt3 ligand, principally plasmacytoid DC ($CD11c^+CD11b^-B220^+$) are formed (81). Plasmacytoid DC are poor inducers of T cell proliferation, but have the unique ability to produce IFN-α in response to viruses. Thus, these DC may be primarily involved in the response to viral infections (7). Murine splenic DC can also be subdivided into different populations on the basis of CD8α expression (238).

It has been suggested that distinct DC subsets drive the differentiation of T cells towards IFN-γ-producing Th1 cells or IL-4-producing Th2 cells (201). Splenic $CD8\alpha^+CD11b^-CD11c^+$ DC have been shown to produce IL-12 and prime a Th1 type response, whereas $CD8\alpha^-CD11b^+CD11c^+$ primed a Th2 type response (140, 175). However, it was subsequently demonstrated that both DC subsets could drive a Th1 response (107). More importantly, recent studies have reported that DC subsets show considerable plasticity in driving the development of Th1/Th2 responses. For example, plasmacytoid DC have been shown to induce a strong Th1 response when stimulated with CpG, but not with LPS, whereas myeloid DC initiated a Th1 response when stimulated with LPS (18). This same study indicated that both types of DC initiated a Th1 response when exposed to high doses of antigen, in contrast low antigen dose induced a Th2 response. This suggests that the nature of the resulting T cell response may be determined by a combination of factors, including the type of DC, the activation state of the DC, the form and dose of antigen and the nature of the invading pathogen.

Heterogeneity of DC subsets can also be observed in humans. Blood is the only source of human DC and therefore only precursor or immature DC can be isolated. Thus, most studies of DC in humans have been conducted using immature DC developed in culture, rather than DC isolated from tissues. Despite this difficulty,

there appear to be distinct developmental pathways for DC in humans – reviewed in (201).

DC have been identified in most tissues and organs. The main varieties are the cutaneous Langerhans cells, circulating DC of peripheral blood, the lymphatic veiled cell and the digitated cells in the spleen and lymph nodes (120, 208). DC have been found in thymus, colon, synovia, peritoneum and lung (124, 171, 200, 248). In the lung, a high presence of DC would be expected, as the lungs are under constant exposure to a variety of inhaled antigens.

Studies in both rodents and humans have demonstrated a vast network of bone marrow-derived DC residing in the mucosa of the nose and large conducting airways, the alveolar lumen and septum and the connective tissues surrounding the blood vessels and pleura (97, 195, 231). The mechanism by which immature DC are recruited to the lung is largely unknown. It is likely that monocyte precursors are recruited from the bloodstream and acquire the immature DC phenotype following migration into tissues and exposure to DC differentiation factors (216). The immunologic incompetence of the bronchial mucosa correlates with the absence of antigen-presenting cells in foetal or newborn bronchial mucosa. In rodents, can observe a progressive co localization of the mucosa with DC occurring during the time between birth and weaning (156). There appears to be a precursor cell which does not express MHC class II and begins to acquire MHC class II expression during the neonatal period. Simultaneously, antigen-presenting cells begin to appear in alveolar tissues.

1.4.2 DC maturation and migration

In vivo, DC exist at different functional and developmental stages. Immature DC reside within the peripheral tissues where they act as sentinels. Following antigen encounter DC mature and migrate to the lymphoid organs where they interact with T cells to initiate an immune response. DC rapidly accumulate at sites of infection or tissue damage, probably due to recruitment of circulating precursors in response to chemokines produced on local inflammation.

Immature DC are very effective at antigen capture and can use several pathways, such as macropinocytosis, receptor-mediated endocytosis and phagocytosis. Receptors involved in antigen uptake include;

- Mannose receptor – a C-type lectin which captures antigens with exposed mannose or fructose residues (188).
- DEC-205 – a C-type lectin which binds unknown carbohydrate ligands (110).
- FcγR types I (CD64) and II (CD32) – involved in uptake of opsonized particles or immune complexes (61).

Internalization via C-type lectins, particularly DEC-205, promotes transport of antigen to specialized antigen processing compartments. For example, antigen internalized via DEC-205 was presented 100 times more efficiently than antigen which did not enter via DEC-205 (110).

Following interaction with antigens, the immature DC undergoes a series of functional and phenotypic changes which results in the transition from antigen-capturing cell to APC. Activation, or maturation, of the DC is linked with their migration to the lymphoid organs. DC activation is initiated in the peripheral tissues and continues to completion during interaction with T cells. A variety of factors promote DC activation, including pathogen-related products (LPS, bacterial DNA and dsRNA), cytokines present in the local environment (IL-1, TNF-α, IL-6) and T cell-derived signals (CD40/CD40L) – for review see (8).

The process of DC activation or maturation is summarised in Figure 1.1 and involves a series of coordinated events including;

- Morphological changes to give characteristic stellate appearance.
- Redistribution of MHC class II molecules from intracellular compartments to the cell surface (174)
- Reduction in antigen capture ability with decreased expression of endocytic/phagocytic receptors.
- Increased expression of costimulatory molecules CD40, CD80 and CD86 (8).
- Production of pro-inflammatory cytokines, such as IL-1, IL-12 and TNF-α (8).
- Release of chemokines, including RANTES, MCP-1, MIP-1α and MIP-1β which attract cells, including DC precursors to re-populate tissues (212)

The factors which mediate DC maturation also trigger their migration to the T cell areas of lymphoid tissues. Maturing DC up-regulate expression of the chemokine receptor CCR7 (212). The ligands for this receptor 6Ckine and MIP-3β are chemoattractants for DC and guide their migration. DC leave peripheral tissues via afferent lymph and travel to lymphoid tissues. On encounter with T cells, either in lymphoid tissues or at site of injury, DC receive additional maturation signals via CD40L (213).

DC in the lung exhibit an immature phenotype, specialized for antigen uptake. In the absence of infection or inflammation there appears to be a continuous migration of DC from the airways into the draining lymph nodes, thus these DC may continuously sample the environment for incoming antigens (96, 127), or may capture bronchial epithelial cells which have become apoptotic as part of normal cell turnover. Under inflammatory conditions, the function of lung DC changes dramatically. In rodents it has been shown that exposure to BCG induces a marked increase in the number and activation status of airway DC (92). The induction of inflammation by invading microbes in the lung accompanies the induction of an

immune response as immature DC which have recognized antigen are induced to migrate via the afferent lymphatics into the draining lymph nodes of the lung and DC are activated to express costimulatory molecules which enable them to stimulate naïve T cells.

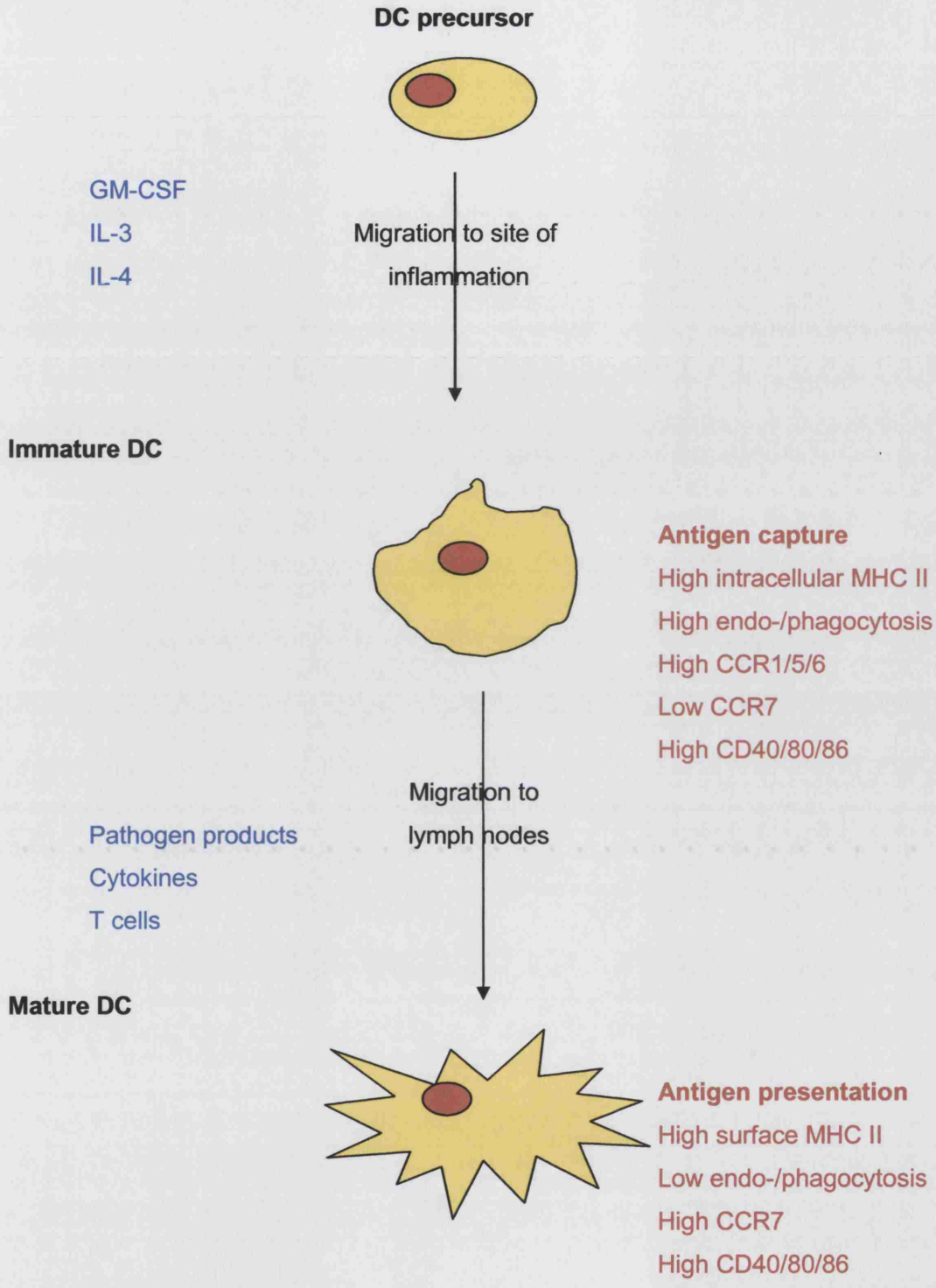

Figure 1.1 Phenotypic changes during DC maturation.

1.4.3 Antigen presentation and T cell stimulation

The unique ability of mature DC to stimulate naïve and effector T cells makes them the most efficient APC. DC can process and present antigen in the context of both MHC class I and II molecules to $CD8^+$ and $CD4^+$ T cells, respectively, and are extremely potent APC requiring only a small number of DC to elicit strong T cell responses (8).

Although DC have been shown to be capable of directly stimulating $CD8^+$ T cells (245), they often require the help of $CD4^+$ T cells. Previously, it was thought that $CD4^+$ and $CD8^+$ T cells recognized antigen on the same APC. However, the current model suggests that DC become primed by interaction with CD40 on $CD4^+$ T cells to activate $CD8^+$ T cells (181). Thus, the DC becomes a bridge between the $CD4^+$ and the $CD8^+$ T cell.

T cell activation requires 2 signals; signal one involves the recognition of peptide-MHC complexes on the APC by antigen-specific TCR, and signal two involves the interaction of costimulatory molecules on the APC with their ligands on the T cell. DC express high levels of MHC molecules and costimulators on the cell surface, and thus can provide both signals required for the stimulation and amplification of T cell responses. Other factors which may be involved in the high capacity of DC to stimulate T cells may include; cytokines present in the local environment, low levels of sialic acid on DC may reduce repulsive force and promote clustering with T cells, similarly DC express high levels of adhesion molecules which may also facilitate clustering and TCR engagement (28). During the DC-T cell interaction, the DC continues the maturation process, receiving additional activation signals from the T cell itself, for example via interaction with CD40 (29).

The final stage in the DC life cycle is apoptosis, probably mediated by T cells clearing the way for the next wave of cells into the lymph nodes via afferent lymph.

This also explains why DC are not observed exiting the lymph node in efferent lymph.

1.4.4 Interaction of DC with mycobacteria

DC have been identified in the airway epithelium and lung parenchyma (98) and have also been shown to accumulate within the granulomas of TB patients (227). As DC are such efficient APC, it is likely that they contribute to the initiation of the protective response against TB.

In addition to Mϕ, mycobacteria have been shown to readily infect DC (93, 104). As discussed in section 1.2.1.2, activated Mϕ can kill intracellular *M. tuberculosis* via the production of RNI (32). A study comparing the ability of activated Mϕ and DC to control *M. tuberculosis* infection demonstrated that although DC can restrict intracellular growth of the bacteria, they are not as efficient as Mϕ at eliminating the infection (16).

A number of studies have indicated that mycobacterial infection of DC is sufficient to induce their activation, as shown by up-regulation of IL-12 and inflammatory cytokines, TNF-α, IL-1 and IL-6 (48, 93, 220), reduced endocytic activity (93), increased surface expression of the costimulatory molecules CD80 and CD86 and the adhesion molecule ICAM-1 (48, 93), increased surface expression of MHC class II molecules (93) and increased elaboration of chemokines including MIP-1α and MIP-1β (50). The activation of DC induced by mycobacterial infection could be enhanced by activating CD40 signalling using agonistic anti-CD40 antibody (51). However, a recent report using human derived DC has suggested that virulent *M. tuberculosis* induces a limited and reversible maturation of DC and may act to inhibit DC function (89). Production of IL-10 during early mycobacterial infection has been observed (93). This may result in a diminished Th1 response via inhibition of IL-12

production, and has also been demonstrated to interfere with DC trafficking and reduce their migration to the draining lymph nodes (49).

Studies comparing the response of Mϕ and DC to mycobacterial infection have shown that DC preferentially produce IFN-γ-inducing cytokines such as IL-12, whereas Mϕ were the main producers of inflammatory cytokines such as TNF-α, IL-1 and IL-6 (79, 95). These reports also demonstrated that Mϕ produce immunosuppressive IL-10, which may be responsible for the lack of IL-12 response by Mϕ. This demonstrates that Mϕ and DC may play different roles during mycobacterial infection, with Mϕ producing high levels of inflammatory cytokines and modulating the granulomatous response, while DC are primarily involved in initiating T cell responses.

M. tuberculosis-infected DC can prime naïve T cells *in vitro* and induce an IFN-γ-producing Th1 type response (79, 83, 95). In addition, mycobacterially-infected DC primed protective T cell responses when administered to mice (104), and the resulting response was of the Th1 type (51). Studies using BCG-infected DC (48) or *M. tuberculosis*-infected DC (220) have also shown that not only do these cells induce a T cell response *in vivo*, but that this response is protective against subsequent challenge with virulent *M. tuberculosis*, achieving levels of protection similar to, or improved upon, that conferred by BCG.

It has recently been shown that *M. tuberculosis* enters DC following binding the C-type lectin receptor DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing non-integrin) via LAM. This appears to be the major receptor on DC for uptake of *M. tuberculosis*, in comparison CR3 and mannose receptor (the major receptors for uptake by Mϕ) seem to play a minor role (217). DC-SIGN serves many functions in DC, including promoting extravasation via binding ICAM-2 on endothelium thus promoting DC migration to inflammatory sites and lymphoid tissues and initiating DC-T cell interactions via binding ICAM-3 on T cells thus

promoting the formation of the immunological synapse. DC-SIGN also serves as an antigen receptor for HIV-1 via gp120 binding and plays a role in the dissemination of HIV by DC – for review see (117). Binding of mycobacteria to DC-SIGN has been shown to suppress DC function (78) by impairing DC maturation and inducing production of IL-10. Thus, binding of mycobacteria to DC-SIGN may promote immunosuppression and contribute to bacterial survival.

1.4.5 DC immunotherapy

The possibility of exploiting the immunoregulatory activity of DC holds enormous promise for their use as immunotherapeutic or immunoprophylactic agents. A DC vaccine has been defined as DC loaded with antigen (e.g. tumor-associated antigen), which on administration is thought to induce an antigen-specific T cell response against the tumor. Inaba, *et al* have demonstrated that injection of DC, charged with antigen *ex vivo*, could sensitize normal mice to protein antigens (208). Most DC vaccines to date have been used to stimulate immune responses, particularly against tumors. Indeed, the first clinical study of a DC vaccine was directed against B cell lymphoma (102).

There is a large body of evidence involving animal models of tumor immunity in which DC loaded with tumor-associated antigens are capable of inducing protective anti-tumor responses and these can often be superior to other vaccination strategies (80). For example, tumor peptide antigens which are presented in the context of MHC class I have been pulsed to DC and can induce protection against lethal challenge by a tumor transfected with a gene encoding the antigen (30). Therapeutic immunity could also be conferred by the transfer of DC presenting tumor antigens into tumor-bearing mice (196). There have also been reports of DC immunization giving significant therapeutic immunity to established tumors.

The immunogenicity of antigens delivered in DC has now been demonstrated in human studies. A single injection of antigen-loaded monocyte-derived DC expanded $CD4^+$ and $CD8^+$ T cell immunity and a single boost months later led to CTL expansion with increased affinity against viral peptide (56). A number of trials have utilized tumor-associated antigen-loaded DC as vaccines in humans. Early studies demonstrated clinical responses in the absence of any significant toxicity (102, 157). More recent vaccination strategies have emphasized the elicited immune responses and have included control antigens for $CD4^+$ and $CD8^+$ T cell responses. It has been shown that T cell immunity to both control antigens (viral peptide and bacterial protein) and melanoma peptide can be induced by vaccination with antigen-pulsed monocyte-derived DC, even in advanced melanoma patients (222).

Experimental DC-based vaccines have also been studied against a variety of infectious diseases, including *Chlamydia* and *Toxoplasma* (20, 163). BCG-pulsed DC (48) and *M. tuberculosis*-pulsed DC (220) transferred into mice have also been shown to confer protection against subsequent challenge with *M. tuberculosis*. Several issues must be considered when developing DC for clinical use. The cells must be generated is sufficient numbers, they should display the morphological, functional and phenotypic characteristics of DC and they should be capable of presenting antigen. Although the numbers of DC in circulation are low, advances have made it possible to generate DC in culture, from various sources including bone marrow and peripheral blood; however for clinical application DC must be cultured in serum-free media and cryopreserved for future use – reviewed in (64). Also when considering DC vaccines the following parameters should be taken into consideration;

- DC subset – the majority of clinical studies so far have utilized *ex vivo* generated monocyte-derived DC (i.e. $CD11c^+$ myeloid DC).

- Optimal DC maturation state – immature DC can be tolerogenic, indeed injection of immature DC led to the inhibition of $CD8^+$ T cell responses to viral peptide (57). Thus, DC maturation state is a critical parameter for the use of these cells in patient immunization.
- DC dose and frequency of injection – in human trials to date DC are given at 2-4 week intervals and at doses of 4-40 million. Higher doses given more frequently may give more intense TCR triggering and so promote T cell priming, but may result in activation-induced death of T cells.
- Antigen loading strategy – the most commonly used method is the loading of MHC molecules with peptides derived from defined antigens. Alternative strategies which give both MHC class I and II epitopes include recombinant proteins, viral vectors, exosomes, plasmid DNA or RNA transfection (72, 80). Another approach is to exploit the capacity of DC to present peptides derived from dead cells (130).
- Vaccine efficacy and quality of immune response – a major difficulty is the identification of immunologic markers to predict the clinical efficacy of immunotherapy. CTL function is often evaluated on peptide-pulsed target cells, but further studies are required.

DC immunotherapy holds promise for not only the treatment of cancer, but also autoimmune diseases, infectious diseases and the prevention of transplant rejection. Early clinical trials have indicated that DC vaccines can induce immune responses in some cancer patients, however study design must be carefully considered and standardized clinical and immunological criteria are required.

1.5 Cross-priming and cross-presentation

The protective response to intracellular pathogens is dominated by T cells which recognize antigen in the context of MHC class I, MHC class II or CD1 molecules (192). It is not surprising that these intracellular pathogens can access the MHC class II pathway, in which antigens are processed within the endosomal system. However, processing of antigen for presentation in association with MHC class I molecules occurs in the cytoplasm and the resulting peptides are transported to the endoplasmic reticulum for loading onto MHC class I molecules, thus it is mainly endogenous proteins which are processed and presented via this pathway. Intracellular pathogens which are able to escape from the phagosome, such as *Listeria monocytogenes* can access the host cell cytoplasm and therefore the MHC class I processing pathway. However, phagosome-contained pathogens such as *mycobacteria*, *Chlamydia*, *Salmonella* and *Leishmania* also induce MHC class I-restricted CD8 responses which participate in the protective response – for review see (192). The mechanisms by which these pathogens access the MHC class I pathway remain unclear, however some possibilities, which are not mutually exclusive are considered in section 1.5.1.

Almost 30 years ago Bevan observed that mice immunized with cells expressing foreign minor histocompatibility antigens mounted an antigen-specific response which was self-MHC-restricted (13). This demonstrated that exogenous pathways are also important in inducing $CD8^+$ T cell responses. This phenomenon was termed cross-priming and suggested that antigen could be transferred from donor cells to recipient cells for presentation in association with MHC class I molecules. The term cross-presentation is commonly used to describe the processing and presentation of exogenously-derived antigens in the MHC class I pathway. It should be noted that the terms cross-priming and cross-presentation are most commonly

used in reference to $CD8^+$ T cells and MHC class I presentation, however $CD4^+$ T cells can also be stimulated in this manner. Figure 1.2 shows a schematic representation of direct and cross-presentation.

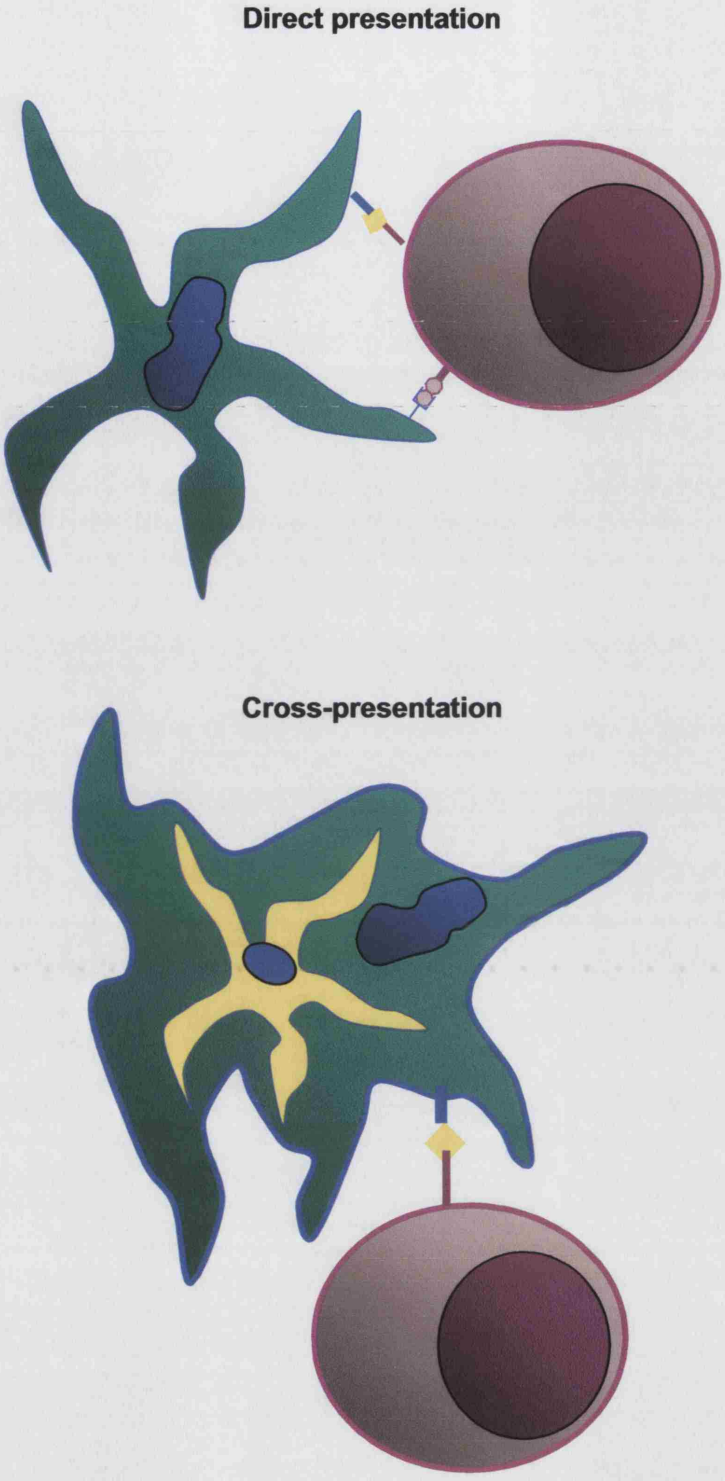

Figure 1.2 Direct and cross-presentation. In direct presentation the APC itself is infected and presents antigen directly to the T cell. In cross-presentation the infected cell/cell fragment is taken up by an uninfected bystander APC which then presents antigen to the T cell.

1.5.1 Routes of cross-priming

Several pathways have been proposed to explain how intraphagosomal microbes may induce MHC class I-restricted responses. The cytosolic route suggests that bacterial factors, such as listeriolysin which allows phagosomal escape of *Listeria monocytogenes* (144), may allow the bacteria or its products to enter the cytoplasm and so access the 'classical' MHC class I pathway. Dependence on transporter associated with antigen processing (TAP) is indicative of this route. The endosomal route proposes that antigens may be loaded into MHC class I molecules out with the endoplasmic reticulum. Peptide exchange may occur due to higher affinity compared to endogenous peptides during trafficking of MHC I molecules through the endosome, or on the cell surface by peptide regurgitation (112).

It has been demonstrated *in vitro* that bacteria with no known mechanism for cytosolic penetration can present bacterial antigen on MHC I (173). This mechanism was found to be resistant to both cyclohexamide and Brefeldin A, suggesting that the classical cytosolic route was not in use. Using bone marrow chimeras reconstituted with bone marrow from TAP-defective donors to investigate cross-priming *in vivo*, it was shown that $CD8^+$ T cell activation in this system was TAP-dependent (103). However, in another *in vivo* study, T cell responses to influenza or lymphocytic choriomeningitis virus (LCMV) were shown to utilize both TAP-dependent and –independent pathways.

Uptake of apoptotic or necrotic cells by uninfected bystander APC represents a potential route of cross-priming during infection with intracellular pathogens. It has been reported that DC can acquire apoptotic influenza-infected monocytes and induce specific $CD8^+$ T cell responses (2). In another study comparing two strains of *Salmonella* which differed in their capacity to induce apoptosis in Mϕ, DC which had engulfed apoptotic infected Mϕ could present bacterial antigens in the context of both MHC I and II (246). However no cross-presentation was observed using a

Salmonella strain which did not induce apoptosis. DC have also been demonstrated to have the capacity to acquire antigen from live cells for cross-presentation (91)

1.5.2 DC as the main cross-priming APC

As DC are specialized to acquire antigen in the peripheral tissues, become activated in response to microbial stimuli, migrate to lymphoid tissues and initiate T cell immunity, they are considered to be the prime candidate for the role of cross-priming APC. It has been shown that bone marrow-derived APC are essential for cross-priming (135, 202). Cross-presentation of antigen derived from apoptotic cells has also been reported to be a property of DC, but not of Mϕ (246), despite the fact that Mϕ could efficiently internalize apoptotic cells. It has been suggested that the unique ability of DC to present antigen derived from apoptotic cells is due to the expression of a unique profile of receptors, particularly $\alpha_v\beta_5$ integrin and CD36 (1). However it has subsequently been reported that $\alpha_v\beta_5$ and CD36 were not essential for cross-presentation of cell-associated antigen (197). Distinctions between DC and Mϕ have also been observed with regard to MHC I-restricted presentation of exogenously delivered protein, such as ovalbumin (OVA) (148) or immune complexes (183). This could be attributable to the fact that DC are more capable of transporting antigen from the endosome to the cytosol than are Mϕ (183).
Using the model of cell-associated OVA being cross-presented by murine splenic DC, it was suggested that only $CD8\alpha^+$ DC were capable of cross-priming $CD8^+$ T cells (53). A subsequent study demonstrated that although $CD8\alpha^+$ DC constitutively cross-present exogenous antigen, $CD8\alpha^-$ DC can also do so following activation, such as via ligation of FcγR (52). Taken together, these data imply that DC represent the predominant APC involved in cross-priming.

1.5.3 The 'detour' pathway of cross-priming

A number of intracellular bacterial pathogens have the ability to induce apoptosis of their host cells, including *Salmonella*, *Listeria*, *Legionella*, *Mycobacteria* and *Chlamydia* (76). As discussed above, apoptotic cells have been demonstrated to be an important source of antigen for cross-priming. A recent study has suggested a novel 'detour' pathway for the presentation of antigen derived from phagosome-contained pathogens (193). This report demonstrated that mycobacterial infection induced apoptosis in Mϕ and that these apoptotic cells were taken up by uninfected bystander DC which processed and presented mycobacterial antigens in the context of both MHC I and CD1 molecules. Apoptosis was essential in this model, as inhibition of apoptosis significantly reduced antigen transfer to DC and subsequent T cell activation.

Apoptosis, or programmed cell death, is a basic physiological function involved in development, growth and tissue homeostasis. Thus in contrast to necrosis, apoptosis is not traditionally associated with inflammation and is considered to be an immunologically inert event. Thus, although apoptotic cells may serve as a source of antigens for cross-priming, there is some controversy as to whether uptake of apoptotic cells can activate DC. Indeed, it was found that although DC could cross-present antigen derived from apoptotic tumor cells, they required a maturation signal for optimal cross-presentation (190). Interestingly, this report also showed that exposure to necrotic cells was sufficient to induce DC maturation, but antigens derived from necrotic cells were not cross-presented. It is now considered that apoptosis is a complex process and that the immunological effects of apoptosis may vary, depending on the circumstances (180). It is possible that infection may lead to a high number of apoptotic cells which cannot be cleared immediately, resulting in secondary necrosis, thus providing a maturation signal to DC. Also, infection-induced apoptosis may induce DC maturation via an alternative route, as microbial

products within apoptotic material may provide a maturation stimulus. Therefore, infection-induced apoptosis, rather than favouring dissemination of the infection may act in favour of the host, promoting cross-presentation and alerting the cell-mediated response to the presence of pathogenic invaders.

Some intracellular microbes, such as *Chlamydia* and *Leishmania* delay apoptosis of their host cell (131). Inhibition of apoptosis by these pathogens is poorly understood, however it has been suggested that they use apoptotic bodies to enter the Mϕ undetected. The 'detour' pathway provides a different view of how delaying apoptosis could be beneficial to the pathogen, by reducing spread of antigen to uninfected DC, T cell activation would be limited and the pathogen may go undetected. This pathway also has implications for future vaccine design, as inducing apoptosis could promote cross-priming of protective T cell responses.

1.6 Aims

This work was conducted to study the interactions between *M. tuberculosis* and primary murine bone marrow-derived DC, which were either unsorted or sorted for $CD11c^+$ DC. The project was divided into three sections;

- A study of the growth of *M. tuberculosis* within DC and the ability of these cells to control the intracellular infection.
- A comparative investigation of the activation of unsorted or $CD11c^+$ DC in response to *M. tuberculosis* infection.
- A study of the ability of murine DC infected with an auxotrophic mutant of *M. tuberculosis* to cross-prime T cells *in vivo* and induce protective immunity.

Chapter 2

Materials and methods

2.1 Bacterial cultures

Mycobacterium tuberculosis H37Rv was grown in 7H9 Middlebrook media (Difco) supplemented with 0.05% Tween 80 and 10% Middlebrook ADC Enrichment (Difco) at 37°C for 14 days, aliquoted and stored at –80°C. Aliquots were thawed and diluted in phosphate buffered saline (PBS) prior to use.

The lysine auxotroph of *M. tuberculosis*, *ΔlysA* ($mc^2$3026) kindly provided by W. Jacobs, AEMC, New York, (169) was grown in 7H9 Middlebrook media (as above) supplemented with 40μg/ml lysine (Sigma) for 14 days, aliquoted and stored at -80°C. Prior to use, cultures were washed three times in PBS, to remove traces of lysine, and resuspended in PBS.

Enumeration of viable bacteria to confirm the multiplicity of infection (MOI) was conducted by plating for viable colony-forming units (CFU) on 7H11 Middlebrook medium (Difco) and incubating for 17-21 days at 37°C.

2.2 Cell culture and conditions

2.2.1 Bone marrow cultures

Bone marrow-derived DC (BMDC) were cultured from bone marrow of C57Bl/6 female mice, as described in (105). Briefly, bone marrow was flushed from femurs and tibia and cells were cultured in cell culture-treated petri dishes at $1x10^7$ cells per plate in 10ml of Iscove's Modified Dulbecco's medium (IMDM; Gibco LifeTech) supplemented with 5% fetal calf serum (FCS), 2mM glutamine (Sigma), 0.1M mercaptoethanol (Sigma) and 10% culture supernatant from Ag8653 myeloma cells transfected with murine granulocyte-monocyte colony-stimulating factor (GM-CSF)

cDNA (20-30U/ml) or recombinant murine GM-CSF (rGM-CSF; Sigma) at 10ng/ml. Cells were incubated at 37°C and 5% CO_2. On day 3 of culture, non-adherent cells were removed and fresh GM-CSF-containing medium was added. On day 6 of culture loosely adherent cells were used as a source of precursor BMDC.
Bone marrow-derived macrophages (BMMϕ) were cultured in the same manner as dendritic cells, with the exception that 30% L929 cell-conditioned medium was used as a source of macrophage colony-stimulating factor (M-CSF) and at day 6 adherent cells were used as a source of BMMϕ.

2.2.2 Immortalized DC line

The temperature-sensitive DC line (tsDC), was kindly provided by Dr B. Stockinger, NIMR (236). The tsDC were cultured in flasks at $1x10^5$ cells/ml in IMDM (Gibco LifeTech) supplemented with 5% FCS and 10mM glutamine (Sigma) and incubated at 34-35°C and 5% CO_2.

2.3 *In vitro* infection of cells with *M. tuberculosis*

Cells (DC or Mϕ) were cultured at $5x10^5$ cells/ml were infected with *M. tuberculosis* (H37Rv) at MOI of 1:1, 5:1 or 10:1 by adding the appropriate volume from the stock bacterial suspension. After 6 hours, cells were washed extensively in fresh media to remove extracellular bacteria and fresh media was added. Cells were then re-cultured at 37°C and 5% CO_2 for the duration of the experiment.

To estimate the percentage of infected cells for each experiment, cells were fixed with 2% paraformaldehyde, and transferred to slides. Slides were stained by the Kinyoun method for acid-fast bacteria.

2.4 Magnetic cell sorting

Cells were enriched by magnetic cells sorting using anti-CD11c microbeads (clone N418) for $CD11c^+$ BMDC, anti-CD4 microbeads (clone GK1.5), or anti-CD8α microbeads (clone 53-6.7) for $CD4^+$ and $CD8^+$ T cells respectively, and a miniMACS separation column (all Miltenyi Biotec) following the manufacturer's instructions. Briefly, day 6 BMDC (for CD11c) or splenocytes isolated from C57Bl/6 mice (for T cells) were incubated with anti-mouse CD16/CD32 ('Fc Block' clone 2.4G2; BD PharMingen) on ice for 15 minutes to prevent non-specific antibody binding. Cells were then labelled with the appropriate microbeads on ice for 30 minutes. Magnetically-labelled cells were washed before passing through a positive selection column (LS^+/VS^+; Miltenyi Biotec). Eluted cells were then passed through a second fresh positive selection column. Purity was assessed by flow cytometry, staining with phycoerythrin- (PE) labelled anti-CD11c (clone HL3), fluoroscein isothiocyanate- (FITC) labelled anti-CD4 (clone H129.19), or FITC-labelled anti-CD8α (clone 53-6.7: all BD PharMingen).

2.5 Fate of *M. tuberculosis* within cells

2.5.1 Intracellular growth of *M. tuberculosis*

Cells were infected with *M. tuberculosis* at MOI 1:1, as described above, (section 2.3) and incubated at 37°C and 5% CO_2. At various time points over the course of 7 days, viable counts of intracellular *M. tuberculosis* were conducted. Cells were lysed in 2% saponin (100µl/ml) for at least 1hour. Viable counts of bacteria released following lysis of the cells was determined by preparing 10-fold serial dilutions in saline and plating onto 7H11 Middlebrook medium (Difco). Plates were incubated at 37°C for 17-21 days before the number of colonies was counted and CFU in the original cell culture calculated.

In experiments where cells were activated with IFN-γ (Sigma) this was added on re-culturing the cells following the 6 hour infection and was maintained at a concentration of 10ng/ml for the duration of the experiment.

In experiments where the competitive inhibitor of inducible nitric oxide synthase (iNOS), nitro-L-arginine methyl ester (L-NAME; Sigma) which is an analogue of arginine and acts to inhibit nitric oxide production, or its inactive isomer nitro-D-arginine methyl ester (D-NAME; Sigma) were used, these were added at the time of infection and were maintained at a concentration of 2mM for the duration of the experiment.

2.5.2 Quantification of nitric oxide production

To quantify the release of nitric oxide (NO) from infected cells, supernatants were collected and double filtered at 0.2µm. The levels of NO produced were measured using the Total Nitric Oxide Assay (sensitivity <1.35µmol/L; R&D Systems) according to the manufacturer's protocol. This assay involves the conversion of

nitrate to nitrite by the enzyme nitrate reductase. The detection of total nitrite is then determined as a coloured azo-dye product of the Greiss reaction that absorbs visible light at 540-570nm.

2.5.3 Cell viability assay

To determine the viability of infected cells, supernatants were collected and double filtered at 0.2μm. Culture supernatants were then run on the Cell Death Detection assay (Roche) as manufacturer's protocol. This uses antibodies directed against DNA and histones and thus can be used to measure apoptotic cell death.

2.6 Phenotypic activation of DC in response to *M. tuberculosis* infection

2.6.1 Cytokine production

To quantify levels of cytokine production from infected DC, culture supernatants were collected at 24 and 48 hours post-infection, double filtered at 0.2μm and stored at -80°C. In these experiments uninfected DC were included as negative controls and as positive controls DC were activated with lipopolysaccharide (LPS; Sigma) at a concentration of 25μg/ml. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure levels of the following cytokines, according to the manufacturer's instructions:

IL-12p40	sensitivity 4pg/ml	(R&D Systems)
IL-12p70	sensitivity 8pg/ml	(Biocarta)
IL-6	sensitivity 8pg/ml	(Biocarta)
IL-10	sensitivity 15pg/ml	(Biocarta)

2.6.2 Flow cytometry

Uninfected, *M. tuberculosis*-infected or LPS-activated DC were washed twice with PBS and incubated with anti-mouse CD16/CD32 ('Fc Block' clone 2.4G2; BD PharMingen) on ice for 15 minutes, to prevent non-specific antibody binding. Cells were then stained on ice for 30 minutes with directly conjugated antibodies: PE-labelled anti-CD11c (clone HL3), FITC-labelled anti-CD80 (clone 16-101A1), FITC-labelled anti-CD86 (clone GL1), FITC-labelled anti-I-A^b (clone AF6-120.1: all BD PharMingen). After staining, cells were washed in PBS and fixed in 4% paraformaldehyde for 2 hours. Following fixation, cells were washed in PBS and resuspended in FACS buffer (PBS + 1% FCS + 0.1% azide) and acquisition was performed on a FACSCalibur (Becton Dickinson) using forward and side scatter characteristics to exclude dead cells. Data was analysed using Win MDI (The Scripps Research Institute, CA).

2.7 *In vivo* response to *M. tuberculosis*-infected DC

2.7.1 Mice

Six to eight week old female C57Bl/6 mice were obtained from breeding colonies maintained under specific pathogen-free conditions in the Division of Biological Services, NIMR.

2.7.2 Infection of DC and immunization procedures

Day 6 BMDC were infected with H37Rv at MOI 10:1 as detailed above (section 2.3). Infection was allowed to continue for 24 hours, then BMDC were washed to remove extracellular bacteria. Infected cells were then irradiated at lethal dose (2.5

megaRads) in the cobalt source at NIMR. Cells were recovered, washed twice and resuspended in AIM-V serum-free media (Gibco LifeTech) to a concentration of $1x10^7$cells/ml. Mice received 250µl ($2.5x10^6$ cells) via the intraperitoneal (IP) route. Mice received three identical injections at 4 week intervals. See Figure 2.1 for illustration of experimental model.

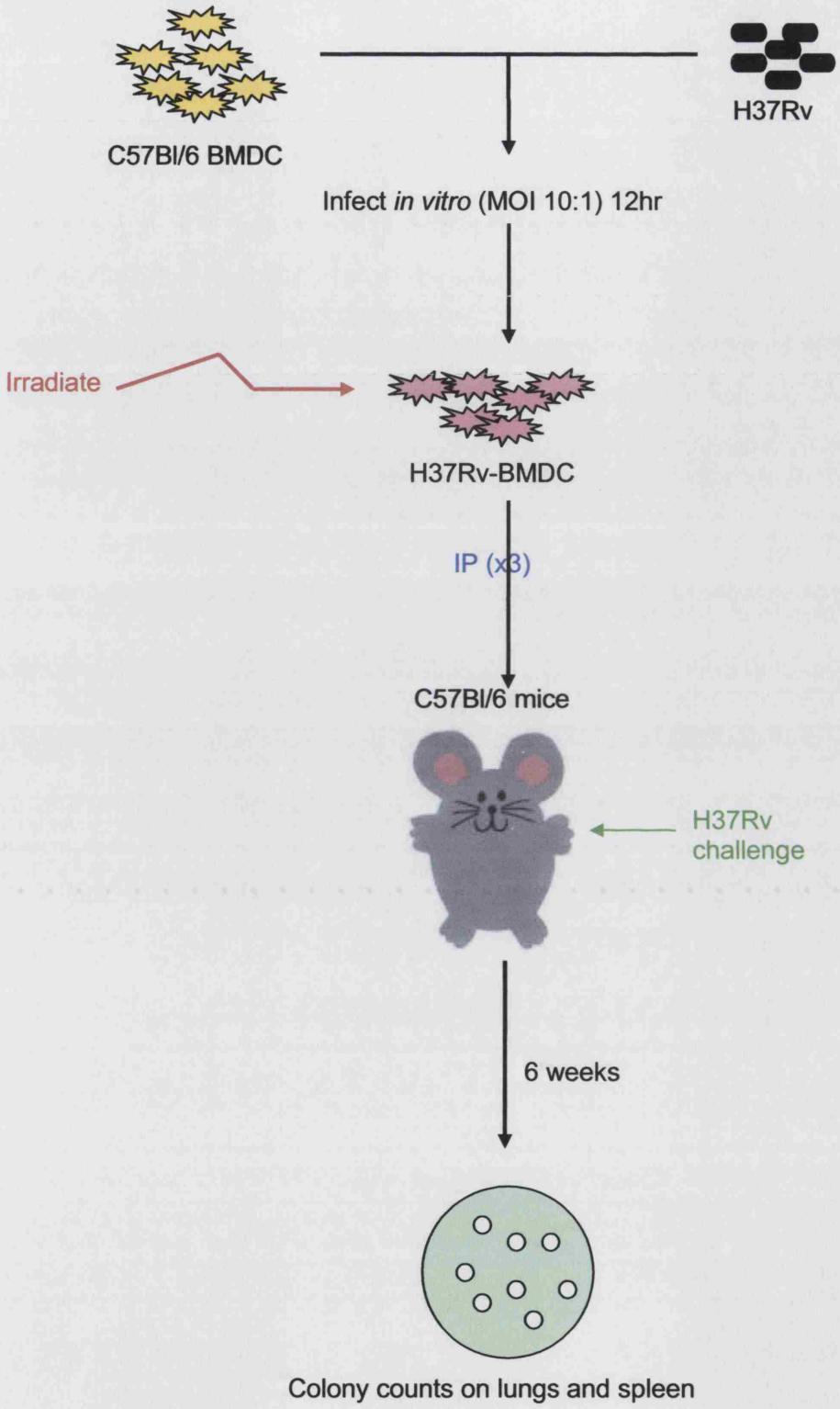

Figure 2.1 Experimental model used to test *in vivo* response to *M. tuberculosis*-infected BMDC

2.7.3 *M. tuberculosis* challenge and protection assay

Six weeks following injection of cells immunized mice were infected via the IV route with approximately 10^6 viable *M. tuberculosis* (H37Rv). Six weeks later, spleens and lungs were harvested and homogenized by shaking in a ribolyser with 2.5mm glass beads for 30 seconds. Serial ten-fold dilutions of the tissue homogenates were prepared in PBS and plated on 7H11 Middlebrook media. Plates were incubated at 37°C for 17-21 days before counting colonies and calculating the number of CFU present in the original tissues.

2.8 Cross-priming *in vivo*

2.8.1 Mice

Six to eight week old female C57Bl/6 were obtained as detailed above (section 2.7.1).

2.8.2 Infection of DC with *ΔlysA* ($mc^2$3026)mutant and immunization procedures

Day 6 BMDC or tsDC were infected with the *ΔlysA* ($mc^2$3026) auxotrophic mutant of *M. tuberculosis* at MOI of 10:1 for 48 hours. Throughout the infection cultures were supplemented with 40μg/ml L-lysine (Sigma). Cells were washed extensively to remove all traces of lysine and free bacteria and resuspended in AIM-V serum-free media (Gibco LifeTech) to a concentration of 10^7 cells per ml. Mice received 250μl ($2.5x10^6$ cells) via the IP route. Mice received three identical injections at 4 week intervals. See Figure 2.2 for illustration of experimental model. In experiments where antibody depletion was conducted mice received 0.1mg via the IP route of

either anti-CD4 (clone YTS 191.1) or anti-CD8 (clone YTS 169.4) 2 days following the final immunization, and then a second identical dose was administered three weeks later.

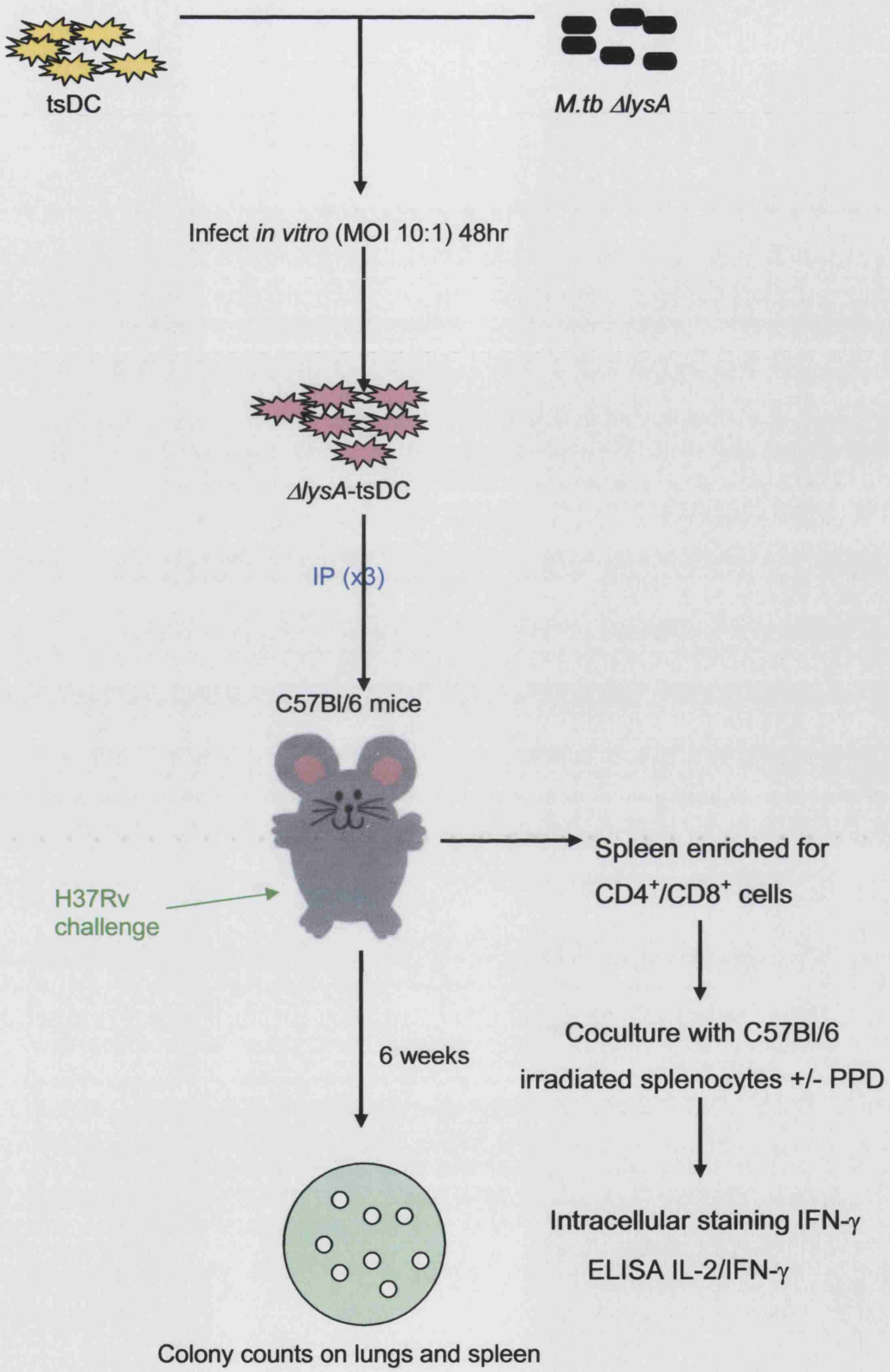

Figure 2.2 Experimental murine model used to test cross-priming *in vivo*.

2.8.3 T cell assays

Spleens were harvested from immunized mice 4 weeks after final injection of infected DC, homogenized via a 70μm cell strainer (Falcon) washed and resuspended in red blood cell lysis buffer (Sigma) for 2 minutes. Cells were then washed and resuspended in AIM-V serum-free media (Gibco Life Tech). Splenocytes were enriched for $CD4^+$ or $CD8^+$ T cells by magnetic cells sorting as detailed in section 2.4. $CD4^+$ or $CD8^+$ T cells were resuspended in fresh media to a concentration of $2x10^6$cells/ml.

Spleens were harvested from naïve female C57/Bl/6 mice, homogenized and red blood cells lysed as above. Splenocytes were resuspended in AIM-V media and irradiated at lethal dose (5000 Rads) in caesium source at NIMR. Splenocytes were recovered, washed and resuspended in AIM-V media to a concentration of $2x10^6$ cells/ml.

In 12 well plates 1ml ($2x10^6$ cells) $CD4^+$ or $CD8^+$ T cells were co-cultured with 1ml ($2x10^6$ cells) irradiated splenocytes. Cells were either untreated, treated with 10μg/ml purified protein derivative (PPD), or 10μg/ml Concavalin A (ConA; Sigma). Cells were incubated at 37°C and 5% CO_2 for 72 hours. Culture supernatants were collected, double filtered at 0.2μm and stored at -80°C after 24 and 72 hours. Culture supernatants were run on commercially available ELISAs for quantification of IL-2 and IFN-γ (both Biocarta; sensitivity 2pg/ml and 8pg/ml respectively).

2.8.4 Intracellular staining

Cells were activated by adding anti-CD3ε (clone 145-2C11) and anti-CD28 (clone 37.51: both BD PharMingen) and GolgiStop (BD PharMingen) which contains monensin and so blocks intracellular transport processes, resulting in the accumulation of cytokine proteins in the Golgi complex, thus enhancing cytokine

staining signals. Cells were incubated at 37°C for 6 hours. Cells were then washed and resuspended in PBS and incubated with anti-mouse CD16/CD32 ('Fc Block' clone 2.4G2; BD PharMingen) on ice for 15 minutes, to prevent non-specific antibody binding. Cells were then stained on ice for 30 minutes with directly conjugated antibodies: FITC-labelled anti-CD4 (clone H129.19) or FITC-labelled anti-CD8α (clone 53-6.7: all BD PharMingen) After staining, cells were washed in PBS and fixed in 4% paraformaldehyde at 4°C for 2 hours. Cells were then washed and resuspended in Cytofix/Cytoperm solution (BD PharMingen) which acts to permeabilize the cells, and incubated at 4°C for 2 hours. Cells were washed in Perm/Wash solution (BD PharMingen) and stained with directly conjugated PE-labelled anti-IFN-γ (clone XMG1.2: BD PharMingen) for 30 minutes on ice. Cells were washed and resuspended in FACS buffer (PBS + 1% FCS + 0.1% azide) and acquisition was performed on a FACSCalibur (Becton Dickinson) using forward and side scatter characteristics to exclude dead cells. Data was analysed using Win MDI (The Scripps Research Institute, CA).

2.8.5 *M. tuberculosis* challenge and protection assay

Six weeks following the final injection of cells immunized mice were challenged with viable *M. tuberculosis* (H37Rv) and counts of viable CFU were conducted as detailed in section 2.7.3.

Chapter 3

Fate of *M. tuberculosis* within dendritic cells.

3.1 Introduction

Following inhalation, the primary target for *M. tuberculosis* is the alveolar Mϕ. However, DC have also been implicated in the phagocytosis of intracellular microbes, such as *Chlamydia* (163), *Salmonella* (143), *Leishmania* (149), *Bordetella* (87), *Borrelia* (65) and *Mycobacteria* (93, 104). Thus DC, in addition to Mϕ, have been shown to function as host cells for a range of intracellular pathogens. As DC are the most efficient antigen-presenting cells, it is likely that they play a pivotal role in the initiation of immune responses against these intracellular pathogens.

Once phagocytosed by the Mϕ, mycobacteria are enclosed within membrane-bound phagosomes (37), and the Mϕ becomes activated to perform anti-microbial effector mechanisms to control the intracellular growth of the bacteria. The fate of mycobacteria within DC is not so clearly defined. It has been shown for both *M. bovis* BCG (104) and *M. tuberculosis* (93, 220) that internalized bacilli are found within membrane-bound vacuoles and are not observed free in the cytoplasm of DC. Studies in murine DC examining both *M. tuberculosis* (16, 220) and *M. bovis* BCG (111) suggest that these cells are capable of controlling the intracellular growth of mycobacteria, although are not as efficient as Mϕ at eliminating the bacilli. Thus, it appears that DC exhibit bacteriostatic activity against intracellular mycobacteria, however the mechanisms by which this occurs are unclear.

In this study initial experiments were carried out to compare the intracellular fate of *M. tuberculosis* within murine bone marrow-derived Mϕ and DC. We then analysed the growth of *M. tuberculosis* within activated murine precursor DC, using both unsorted BMDC and $CD11c^+$ sorted DC populations, and investigated whether this intracellular growth correlated with the production of nitric oxide by DC. Finally, the

effect of *M. tuberculosis* infection on the viability of the DC themselves was examined.

3.2 Comparison of *M. tuberculosis* growth in DC and Mϕ.

It is well established that *M. tuberculosis* can survive and replicate within Mϕ. This experiment was conducted to examine the viability of the bacteria within DC. Murine BMMϕ and BMDC were compared in their ability to support the intracellular growth of mycobacteria. Cells were cultured at $5x10^5$cells/ml and infected with wild type *M. tuberculosis* (H37Rv) at MOI 1:1 for 6 hours. Acid-fast staining showed that following infection approximately 50-70% of cells in each culture were infected. Cells were incubated for 7 days post-infection and were lysed on days 0, 4 and 7 when viable counts of *M. tuberculosis* were carried out.

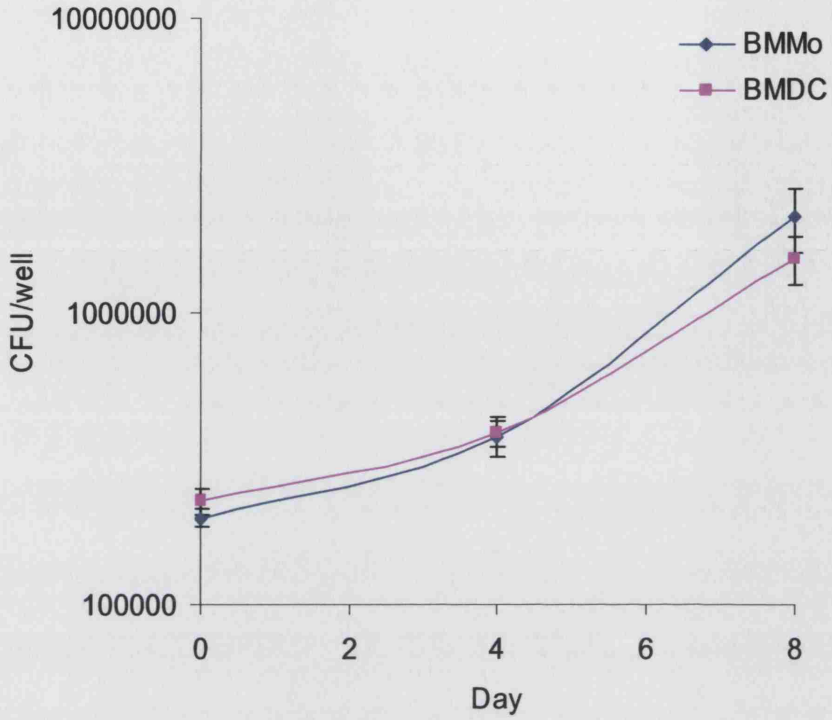

Figure 3.1 Growth of *M. tuberculosis* H37Rv in BMMϕ and BMDC. Murine BMMϕ or BMDC were infected with H37Rv (1:1) for 6 hours. CFU were measured over the course of 7 days to monitor intracellular growth of the bacterium. Results show the mean CFU +/- SEM of 3 independent experiments. At no time point was there a significant difference between growth in these 2 cell populations as measured by Student's t-test.

The results (Figure 3.1) demonstrate that DC were at least as efficient as Mϕ in uptake of *M. tuberculosis* and that there was no significant difference in intracellular viability of the bacteria between the two cell populations. Thus, suggesting that DC can support the intracellular survival and replication of *M. tuberculosis* equally as efficiently as Mϕ.

3.3 Growth of *M. tuberculosis* in activated DC.

Following infection with *M. tuberculosis*, Mϕ become activated to induce anti-mycobacterial effector mechanisms, such as the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), to control the intracellular growth of the bacteria. In order to determine whether activated DC could control intracellular growth, murine precursor BMDC were treated with IFN-γ and the growth of intracellular *M. tuberculosis* was monitored over the course of 7 days, as before.

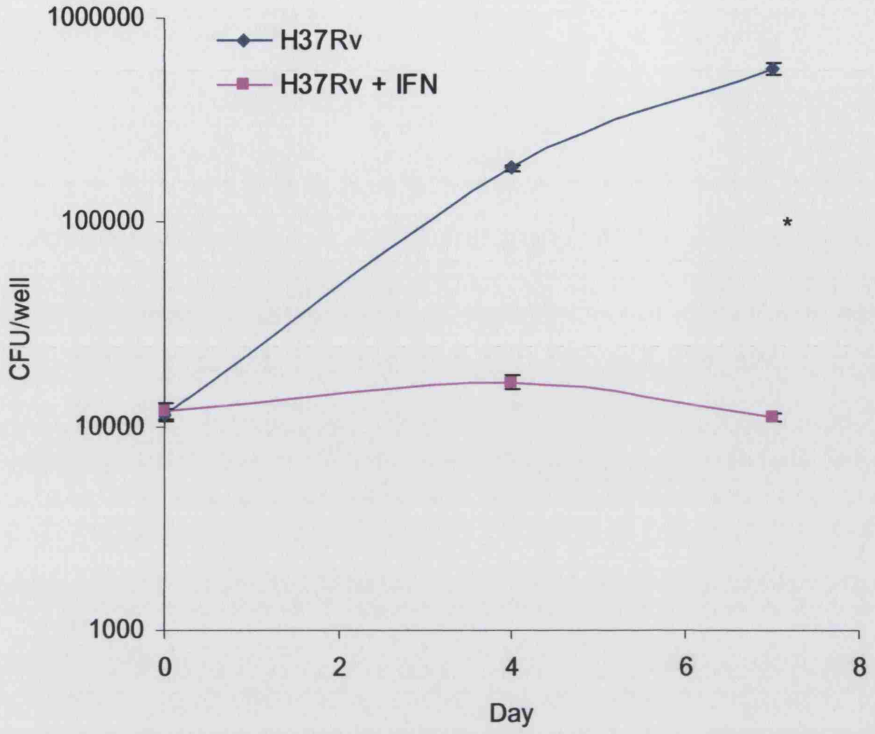

Figure 3.2 Growth of *M. tuberculosis* H37Rv in IFN-γ-activated BMDC. Murine BMMϕ or BMDC were infected with H37Rv (1:1) for 6 hours and where present IFN-γ was maintained at a concentration of 10ng/ml for the duration of the experiment. CFU were measured over the course of 7 days to monitor intracellular growth of the bacterium. Results show the mean CFU +/- SEM of 4 independent experiments. * $P< 0.01$ as measured by Student's t-test.

Figure 3.2 shows that there was a significant reduction in intracellular growth within IFN-γ-treated DC, thus indicating that activated DC can indeed control intracellular growth of *M. tuberculosis*.

RNI, particularly nitric oxide (NO), have been shown to have a particularly profound effect in suppressing growth and killing intracellular *M. tuberculosis* within activated Mϕ (32). As murine DC have previously been shown to be capable of producing NO (17), we investigated production of NO in response to *M. tuberculosis* infection. NO production was measured in cell supernatants from uninfected and H37Rv-infected

BMDC which were either unactivated or treated with IFN-γ as previously. Results are shown in Figure 3.3.

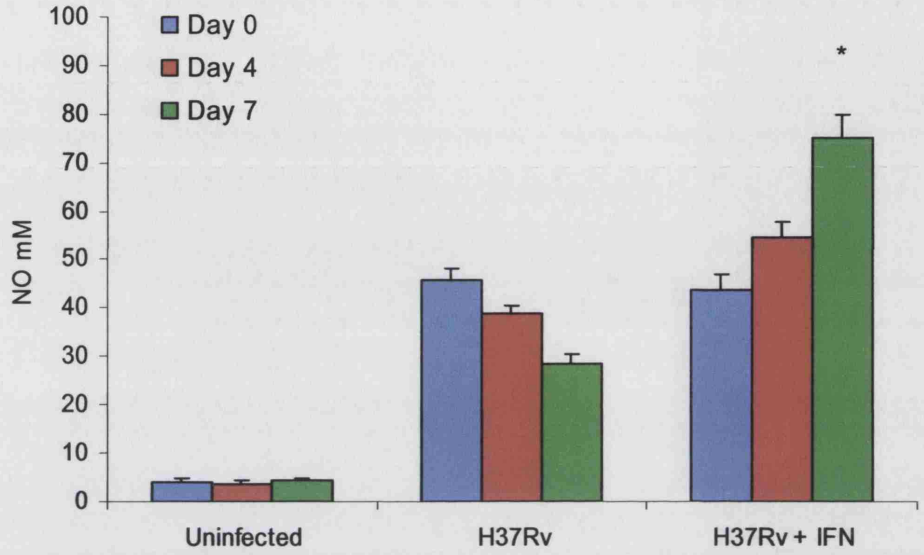

Figure 3.3 NO production by *M. tuberculosis*-infected BMDC.
Murine BMMϕ or BMDC were infected with H37Rv (1:1) for 6 hours and where present IFN-γ was maintained at a concentration of 10ng/ml for the duration of the experiment. Supernatants were collected and NO measured by ELISA. Results show mean +/- SD of 4 independent experiments.
* P< 0.04 as measured by Student's t-test.

DC produced NO in response to *M. tuberculosis* infection, as compared to uninfected controls. In unactivated DC, NO levels decreased over the course of the experiment, however in IFN-γ-activated DC a steady increase in NO production was observed, and this appears to correlate with the growth suppression observed in Figure 3.2. As can be seen in Figure 3.2 the day 4 time point shows almost a 1-log difference between unactivated and IFN-γ-activated DC, however the difference in NO production at this time point (Figure 3.3) does not appear to be so significant. This may suggest the presence of an alternative mechanism of controlling

intracellular growth of the bacteria (e.g. ROI production). However, by day 7 the production of NO by IFN-γ-activated DC has increased significantly in response to infection. Thus, IFN-γ-activated DC are capable of controlling the intracellular growth of *M. tuberculosis* and this is associated with increased production of NO.

3.4 Comparison of *M. tuberculosis* growth in BMDC and CD11c$^+$ sorted DC.

The results observed in section 3.2 and 3.3 demonstrate that DC behave in a similar manner to Mϕ with respect to controlling intracellular growth of *M. tuberculosis*. As the DC used in these experiments were derived from bone marrow cultures grown in the presence of GM-CSF, (section 2.2.1) it was possible that this may be due to the presence of contaminating Mϕ within these cultures. To address this issue, these experiments were repeated using a population of BMDC positively selected on the basis of CD11c expression. Day 6 precursor BMDC were enriched for CD11c$^+$ cells by magnetic cell sorting, (described in section 2.4). Analysis of sorted cells by flow cytometry showed them to be 93-95% pure, as shown in Figure 3.4.
Experiments investigating intracellular growth of *M. tuberculosis* and NO production were repeated comparing unsorted BMDC and CD11c$^+$ DC populations. As previously, cells were either unactivated or treated with IFN-γ at a concentration of 10ng/ml and growth or NO production was measured over the course of 7 days. Results are shown in Figure 3.5 and Figure 3.6.
We observed no significant difference in either intracellular growth of *M. tuberculosis*, or NO production in response to infection between the two cell populations. Also, results obtained were similar to those observed in earlier

experiments. These results strongly suggest that DC can control *M. tuberculosis* intracellular growth, and produce NO in response to mycobacterial infection.

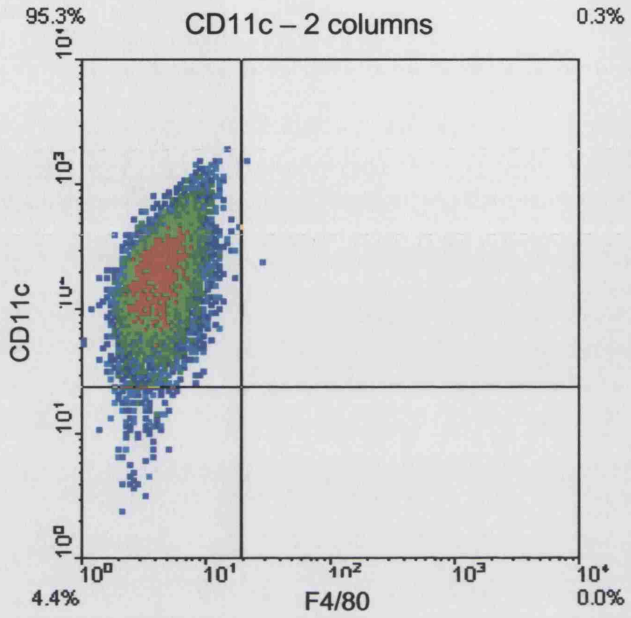

Figure 3.4 Purity of CD11c$^+$ sorted DC. Cells were analysed by flow cytometry following MACs sorting. Cells were stained with PE-labelled anti-CD11c and FITC-labelled anti-F4/80 (Mϕ marker). Results show percentage of total cells and represent a typical experiment. Purity observed was consistently 93-95%.

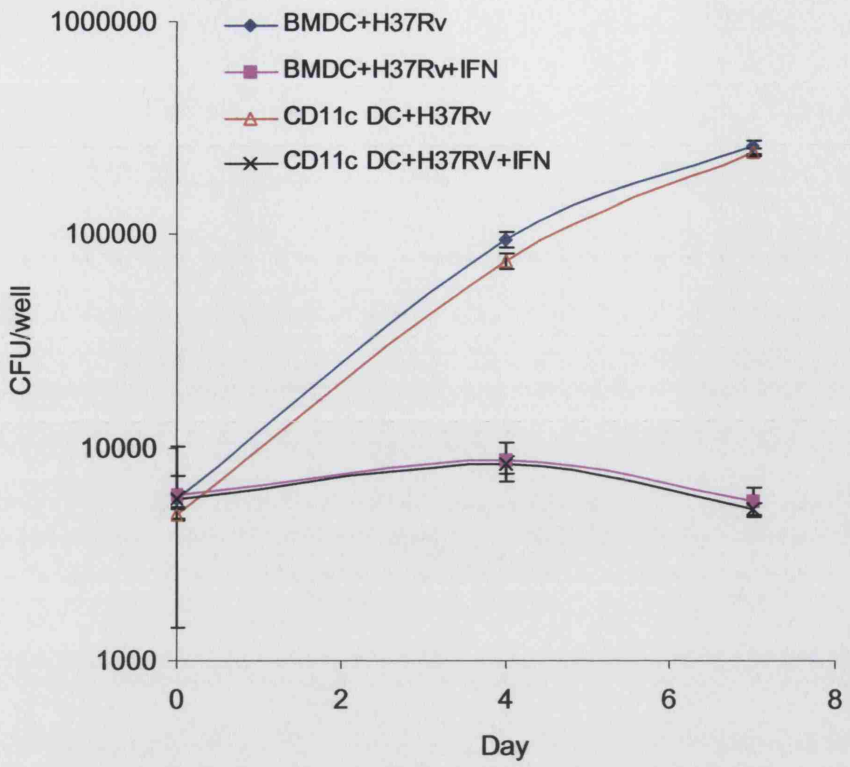

Figure 3.5 Growth of *M. tuberculosis* H37Rv in unsorted BMDC and CD11c$^+$ sorted DC. BMDC or CD11c$^+$ DC were infected with H37Rv (MOI 1:1) for 6 hours and culture was maintained for 7 days in the presence/absence of 10ng/ml IFN-γ.Results show mean CFU +/- SEM of 2 independent experiments.

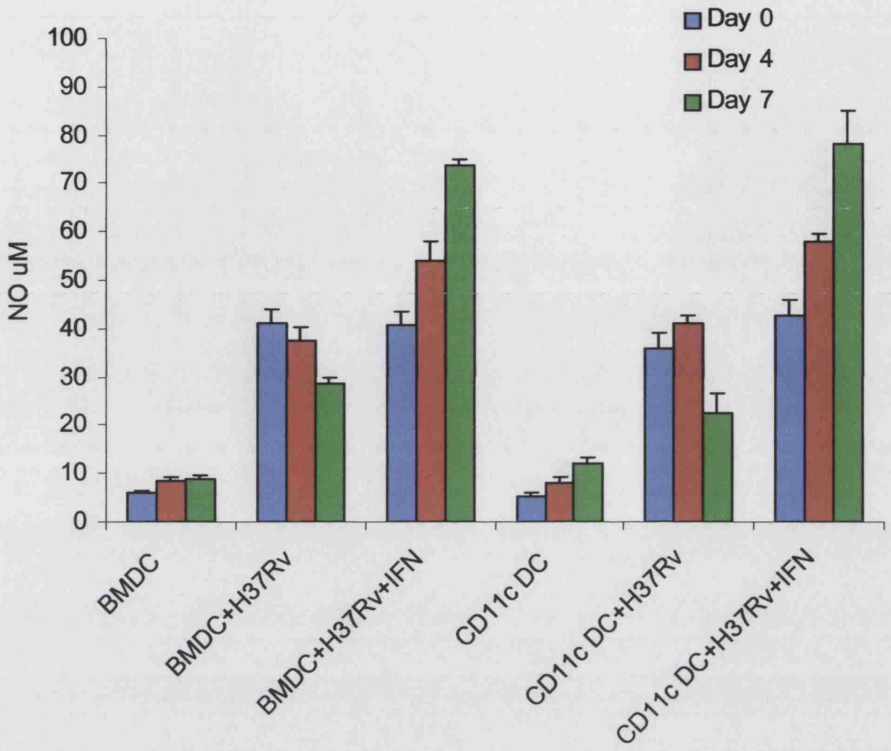

Figure 3.6 NO production in unsorted BMDC and sorted CD11c$^+$ DC infected with *M. tuberculosis*. BMDC or CD11c$^+$ DC were infected with H37Rv (MOI 1:1) for 6 hours and culture was maintained for 7 days in the presence/absence of 10ng/ml IFN-γ. Supernatants were collected and NO measured by ELISA. Results show mean +/- SD of 2 independent experiments. There was no significant difference between the 2 cell populations as measured by Student's t-test.

3.5 Effect of iNOS inhibitor on *M. tuberculosis* growth within DC.

Previous experiments have shown that activated DC can control intracellular growth of *M. tuberculosis*, and that these cells produce NO in response to *M. tuberculosis* infection. Although NO production appears to correlate with the observed growth

inhibition, these experiments provide no information as to whether NO production is responsible for the control of intracellular growth. In order to investigate this we used the competitive inhibitor of inducible nitric oxide synthase (iNOS) nitro-L-arginine methyl ester (L-NAME; analogue of L-arginine; inhibits NO production). As a negative control D-NAME (inactive isomer) was also included. Figure 3.7 shows the effect of this inhibitor on growth of *M. tuberculosis* within activated $CD11c^+$ DC and Figure 3.8 shows the associated effects on NO production.

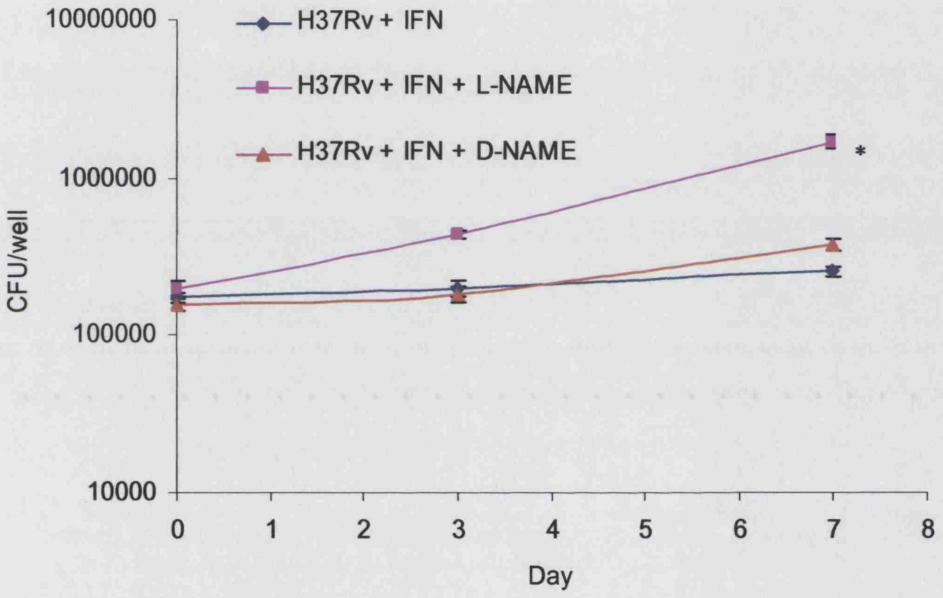

Figure 3.7 Growth of *M. tuberculosis* H37Rv in sorted $CD11c^+$ DC in the presence of iNOS inhibitor. $CD11c^+$ DC were infected with H37Rv (MOI 1:1) for 6 hours and maintained in culture for 7 days. IFN-γ was maintained at 10ng/ml and L-NAME/D-NAME at 2 mM for the duration of the experiment. Results show mean CFU +/- SEM of 2 individual experiments. * $P<0.001$ as measured by the Student's t-test.

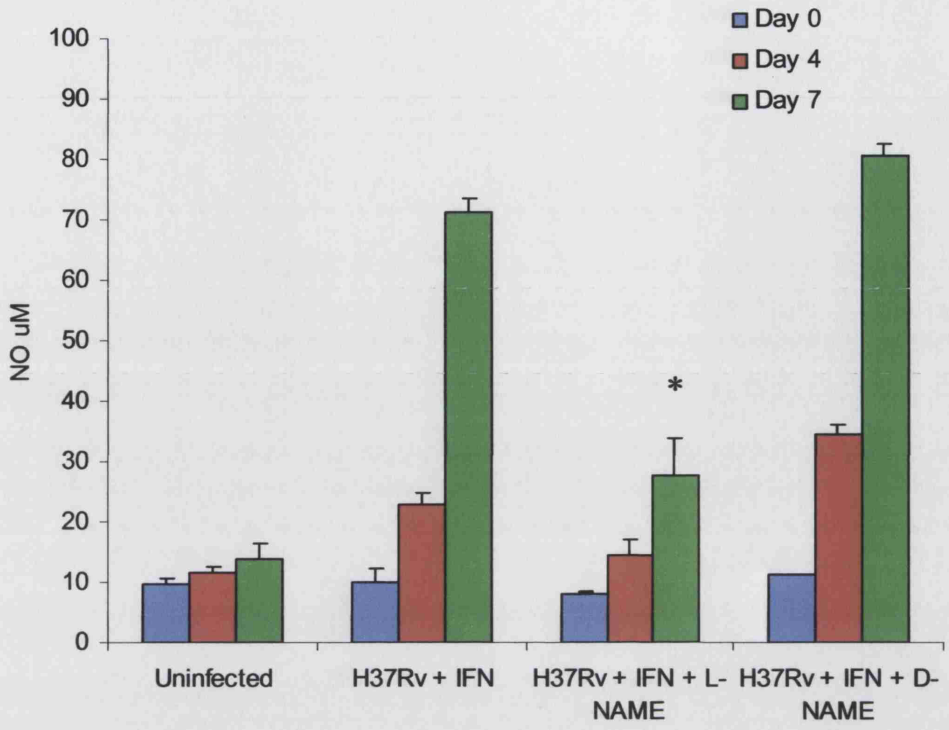

Figure 3.8 Effect of iNOS inhibitor on NO production by *M. tuberculosis*-infected CD11c$^+$ DC. CD11c$^+$ DC were infected with H37Rv (MOI 1:1) for 6 hours and maintained in culture for 7 days. IFN-γ was maintained at 10ng/ml and L-NAME/D-NAME at 2 mM for the duration of the experiment. Results show mean +/- SD 2 independent experiments.
* $P<0.05$ as measured by the Student's t-test.

The addition of L-NAME resulted in failure of DC to control the intracellular growth of *M. tuberculosis*, almost completely abrogating the effects of IFN-γ-induced activation, as CFU returned to levels similar to that observed in non-activated DC. This effect correlated with the expected reduction in NO levels in cells treated with L-NAME. Cells treated with the inactive isomer D-NAME showed no significant difference from activated DC in either control of intracellular growth or NO production. Thus these experiments suggest that IFN-γ-activated DC control *M. tuberculosis* infection and this is, at least partially, due to increased NO production.

3.6 Effect of *M. tuberculosis* infection on DC viability.

In all the experiments conducted in this chapter, cells were infected with H37Rv at MOI of 1:1 for 6 hours before removing extracellular bacteria and re-culturing in fresh media for the duration of the experiment. At this MOI acid-fast staining showed that 50-70% of cells were infected after 6 hours. If the MOI was increased to 5:1 or 10:1 the percentage of infected cells was much higher (up to 85%). However, as shown in Figure 3.9, this was associated with a substantial reduction in DC viability after 48 hours of culture. Thus, by increasing the MOI a larger percentage of infected cells was obtained, however this also resulted in a substantial loss of DC viability.

Cell viability was measured using an ELISA which detects histone-associated DNA fragments in cell supernatants and thus can be used to detect apoptotic cell death. However, a limitation of this assay is that the final measurement is absorbance, which is difficult to relate to actual levels of cell death within the culture. Thus, this assay provides a qualitative measurement only. The constraints of time did not permit these experiments to be repeated using an alternative assay

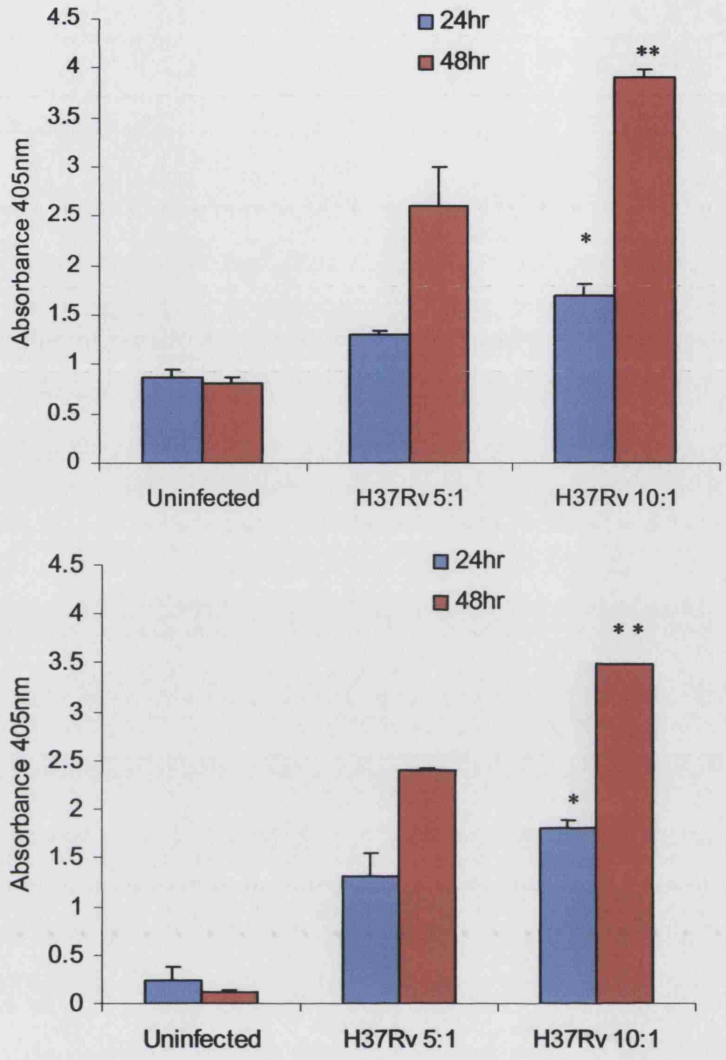

Figure 3.9 Effect of *M. tuberculosis* H37Rv infection on DC viability. Results show mean +/- SD of triplicate wells and are representative of 2 experiments. (**A**) C57Bl/6 BMDC * $P<0.03$ ** $P<0.0009$. (**B**) $CD11c^+$ DC * $P<0.009$ ** $P<0.003$ as measured by the Student's t-test.

Chapter 4

Response of DC to *M. tuberculosis* infection

4.1 Introduction

In the previous chapter it was demonstrated that *M. tuberculosis* infects and can grow in DC. In the peripheral tissues DC are present in an immature state, are unable to stimulate T cells but are specialized for antigen capture. Following interaction with microbial products, DC are activated to develop a mature phenotype, in which they increase the expression of costimulatory and antigen presenting molecules on the cell surface and secrete increased levels of cytokines (8). In this activated state DC migrate to the lymphoid tissues and are able to prime antigen-specific T cells.

In addition to being efficient APC, DC also modulate the immune response by controlling the differentiation of T cells into Th1 or Th2 type cells (162). The production of IL-12 by DC as a rapid response to intracellular pathogens is believed to be a major determinant in establishing a Th1 type response, thus providing an important link between the innate and acquired responses (224, 225). This immunomodulatory function of DC is likely to be of importance during mycobacterial infection where a Th1 type response is required for protection. It is important however to consider that other factors are likely to be involved in determining the type of response stimulated, such as form and dose of antigenic stimulus and the expression of different costimulators.

Previous studies have shown that human DC take up *M. tuberculosis* and differentiate towards an activated phenotype (93). This involved the production of both Th1 (IL-12, IL-1 and TNF-α) and Th2 (IL-6 and IL-10) type cytokines and the increased expression of antigen presenting and costimulatory molecules on the DC surface. Similar results were reported in BCG-infected primary murine DC (48) and in a DC line infected with *M. tuberculosis* (220). The ability of DC to produce both Th1 and Th2 type cytokines in response to infection emphasizes that the factors

influencing polarization of the response may be complex and is likely to be regulated at many levels.

The experiments described in this chapter were aimed at investigating the activation state of primary murine bone marrow-derived precursor DC in response to *M. tuberculosis* infection and comparing the responses observed with unsorted BMDC and those sorted on CD11c.

4.2 Cell surface expression of CD40, CD80, CD86 and MHC class II by DC in response to *M. tuberculosis* infection

In these experiments DC were infected with H37Rv at an MOI of 5:1 or 10:1 for 6 hours, extracellular bacteria were then removed and the DC cultured in fresh media for 24 or 48 hours before performing flow cytometry; cell surface expression of CD40, CD80 (B7.1), CD86 (B7.2) and MHC class II (I-A^b) was then characterized as described in section 2.6.2. Unsorted BMDC were gated on forward and side scatter characteristics (FSC and SSC); for the $CD11c^+$ DC groups cells were also gated on CD11c expression. Uninfected and LPS-treated cells were included in these experiments as negative and positive controls, respectively. Results are illustrated in Figures 4.1 and 4.2 for unsorted BMDC and Figures 4.3 and 4.4 for $CD11c^+$ DC. Results shown are representative of 3 independent experiments.

These experiments were also originally conducted including a group in which cells were infected at an MOI of 1:1. These results were not included as this MOI resulted in no significant change in the surface marker expression as measured up to 48 hours post-infection.

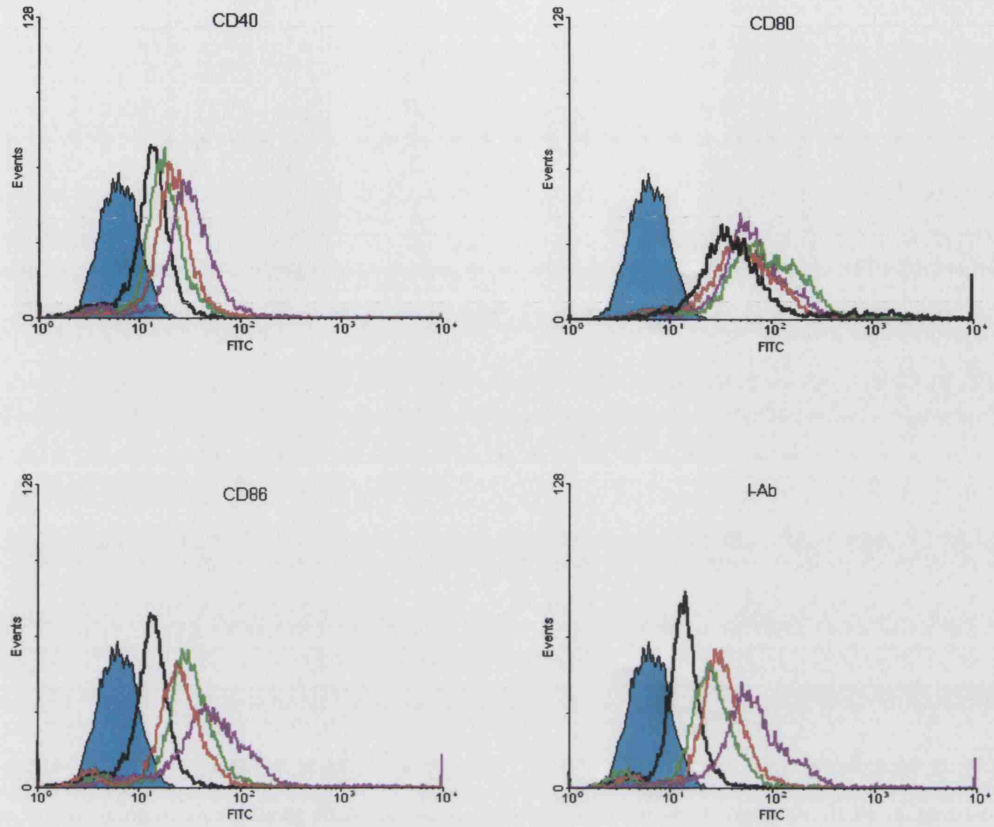

Figure 4.1 Expression of CD40, CD80, CD86 and MHC class II (I-Ab) on *M. tuberculosis*-infected BMDC at 24 hours post-infection. Cells were gated on FSC and SSC. Histograms show the isotype control (pale blue filled), uninfected BMDC (black line), *M. tuberculosis* H37Rv 5:1 (green line), *M. tuberculosis* H37Rv 10:1 (red line) infected BMDC and LPS-stimulated BMDC (purple line).

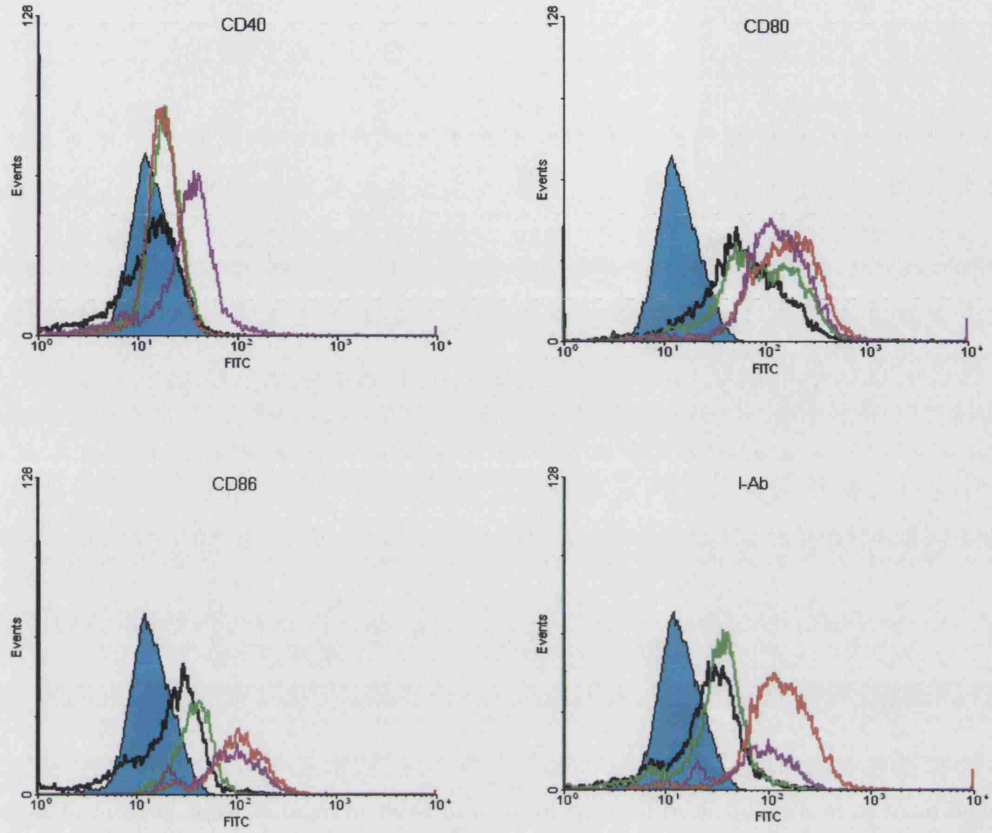

Figure 4.2 Expression of CD40, CD80, CD86 and MHC class II (I-Ab) on *M. tuberculosis*-infected BMDC at 48 hours post-infection. Cells were gated on FSC and SSC. Histograms show the isotype control (pale blue filled), uninfected BMDC (black line), *M. tuberculosis* H37Rv 5:1 (green line), *M. tuberculosis* H37Rv 10:1 (red line) infected BMDC and LPS-stimulated BMDC (purple line).

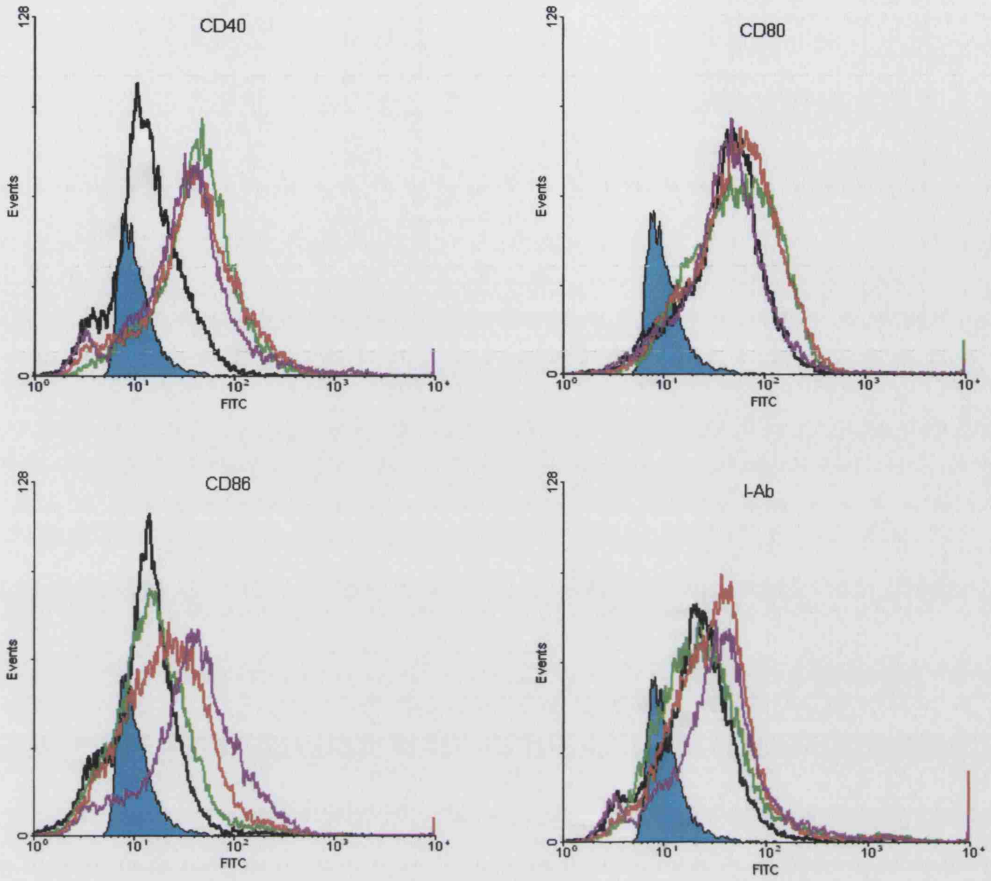

Figure 4.3 Expression of CD40, CD80, CD86 and MHC class II (I-Ab) on *M. tuberculosis*-infected CD11c^{+} BMDC at 24 hours post-infection. Cells were gated for CD11c^{high} expression. Histograms show the isotype control (pale blue filled), uninfected BMDC (black line), *M. tuberculosis* H37Rv 5:1 (green line), *M. tuberculosis* H37Rv 10:1 (red line) infected BMDC and LPS-stimulated DC (purple line).

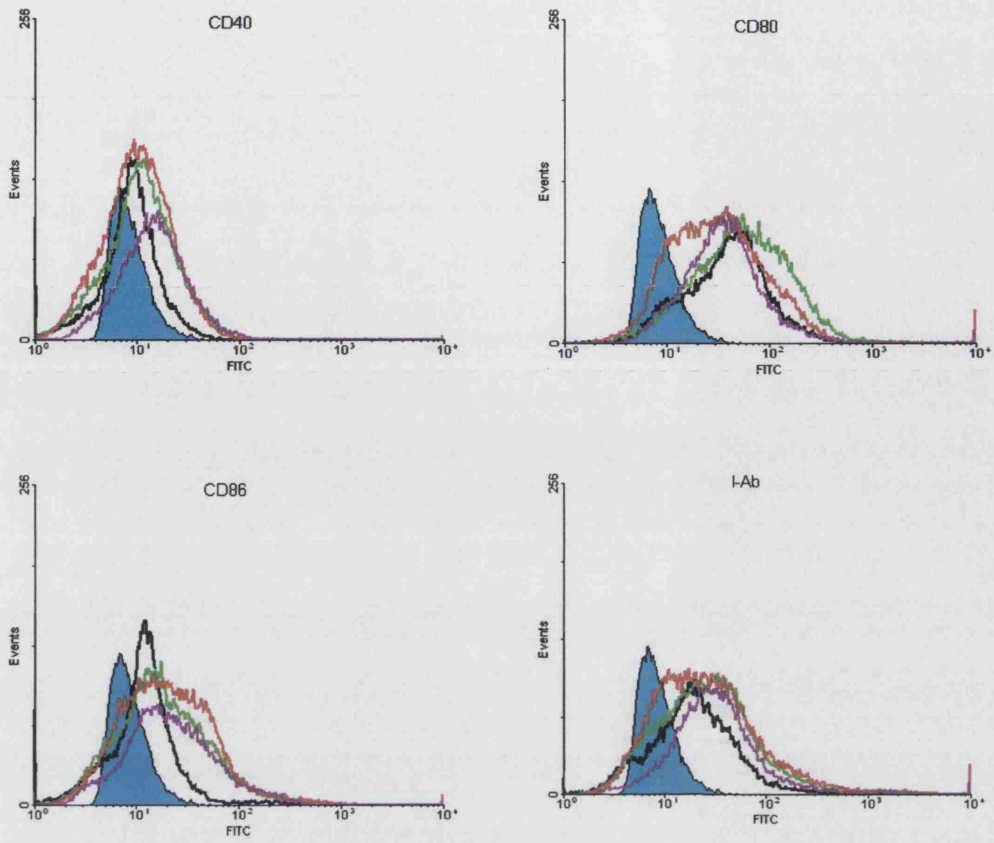

Figure 4.4 Expression of CD40, CD80, CD86 and MHC class II (I-Ab) on *M. tuberculosis*-infected CD11c$^+$ BMDC at 48 hours post-infection. Cells were gated for CD11c^{high} expression. Histograms show the isotype control (pale blue filled), uninfected BMDC (black line), *M. tuberculosis* H37Rv 5:1 (green line), *M. tuberculosis* H37Rv 10:1 (red line) infected BMDC and LPS-stimulated DC (purple line).

As shown in Figures 4.1 to 4.4, expression of CD40, CD80 (B7.1), CD86 (B7.2) and MHC class II (I-A^b) in both DC populations was up-regulated in response to *M. tuberculosis* infection, particularly after 48 hours culture at the higher ratio of infection.

4.3 Cytokine production by unsorted BMDC and sorted $CD11c^+$ DC in response to *M. tuberculosis* infection

The production of IL-6, IL-10, IL-12p40 and IL-12p70 (bioactive molecule) by unsorted BMDC and sorted $CD11c^+$ DC in response to *M. tuberculosis* infection was quantified by ELISA at 24 and 48 hours post-infection. Again, uninfected and LPS-treated cells were included as controls. Purification of $CD11c^+$ DC was carried out by MACS (as described in section 2.4).

Figure 4.5 shows that production of IL-6 is increased in response to infection with *M. tuberculosis* in both unsorted BMDC and $CD11c^+$ DC, however $CD11c^+$ DC produced much higher amounts than the unsorted population. Figure 4.6 shows that $CD11c^+$ DC produced IL-10 in response to *M. tuberculosis* infection, but by 48 hours post-infection levels were reduced. However, unsorted BMDC appear to produce relatively high background levels of IL-10, as production was observed even by uninfected cells at a level similar to that observed in response to *M. tuberculosis* or LPS. IL-12p40 (Figure 4.7) was produced by $CD11c^+$ DC in response to *M. tuberculosis* infection, with levels increasing over the course of the experiment. Unsorted BMDC produced only very low levels of IL-12p40 in comparison. Importantly, IL-12p70 (Figure 4.8) levels were undetectable in infected unsorted BMDC, while $CD11c^+$ DC produced significant levels of IL-12p70 in response to infection.

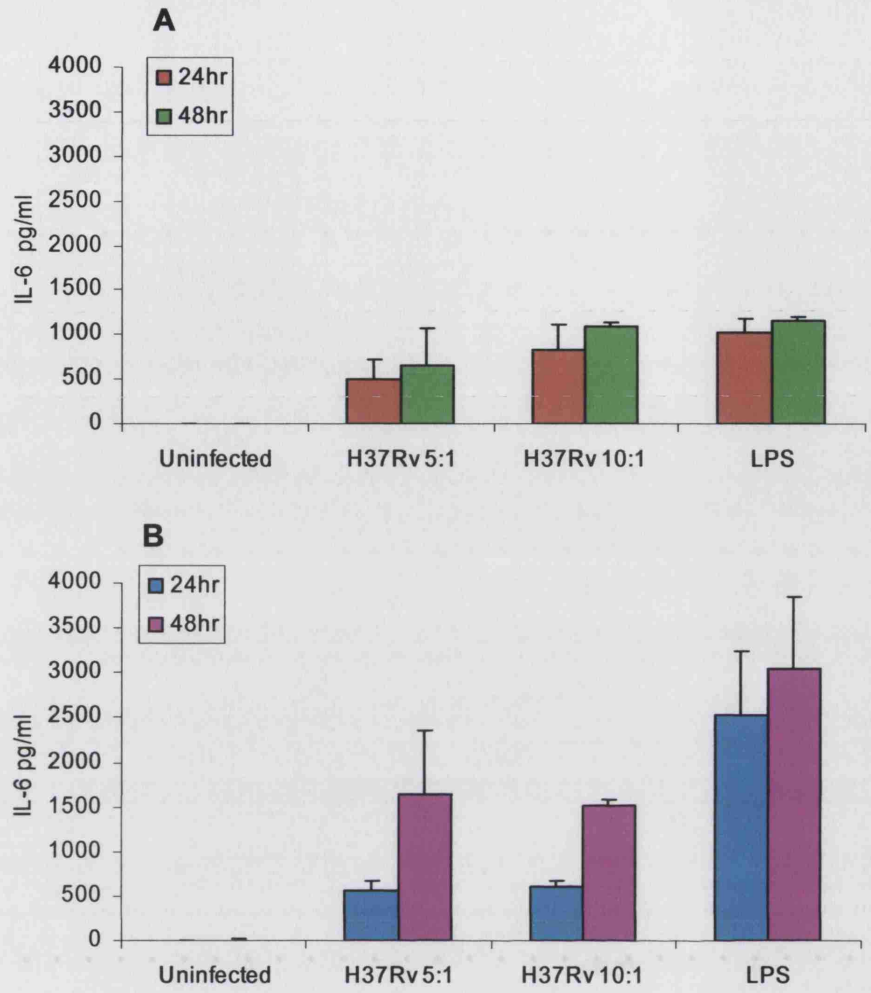

Figure 4.5 IL-6 production in response to *M. tuberculosis* infection in unsorted BMDC and sorted CD11c$^+$ BMDC. (A) Unsorted BMDC. **(B)** CD11c$^+$ DC. Cells were cultured *in vitro* for 24 or 48 hours and supernatants were collected and analyzed by ELISA for the presence of IL-6. Results show the mean +/- SD of 2 independent experiments.

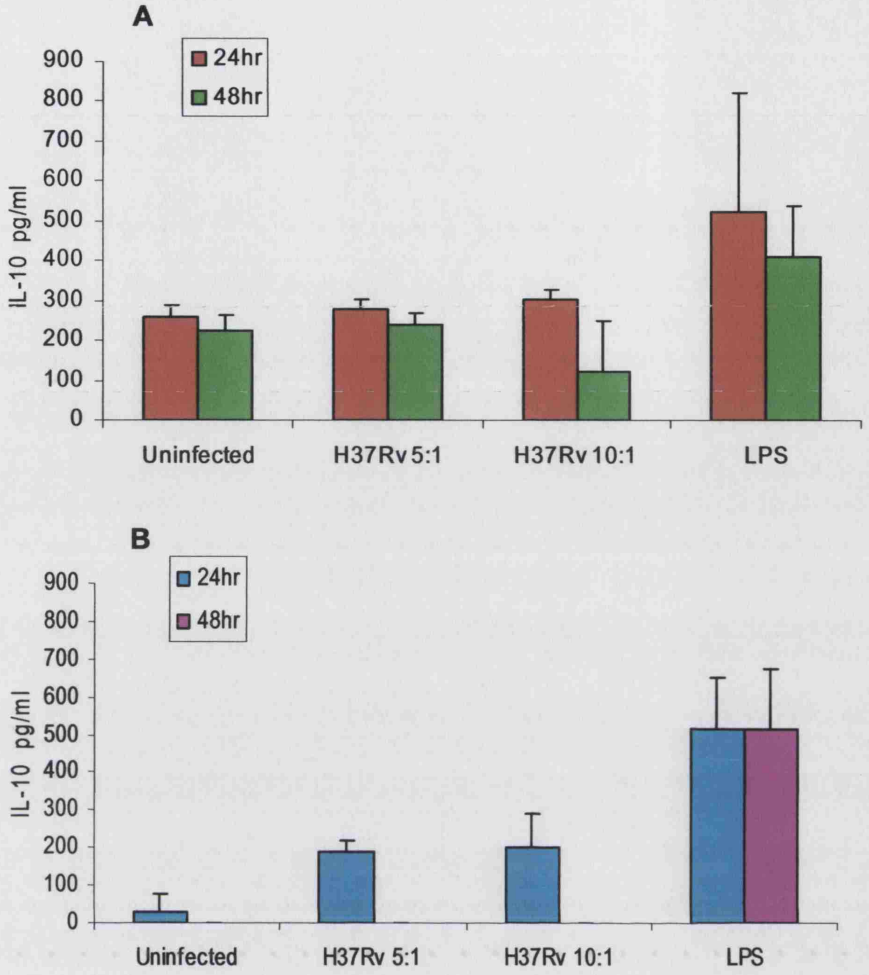

Figure 4.6 IL-10 production in response to *M. tuberculosis* infection in unsorted BMDC and sorted CD11c$^+$ BMDC. (A) Unsorted BMDC. **(B)** CD11c$^+$ DC. Cells were cultured *in vitro* for 24 or 48 hours and supernatants were collected and analyzed by ELISA for the presence of IL-10. Results show the mean +/- SD of 2 independent experiments.

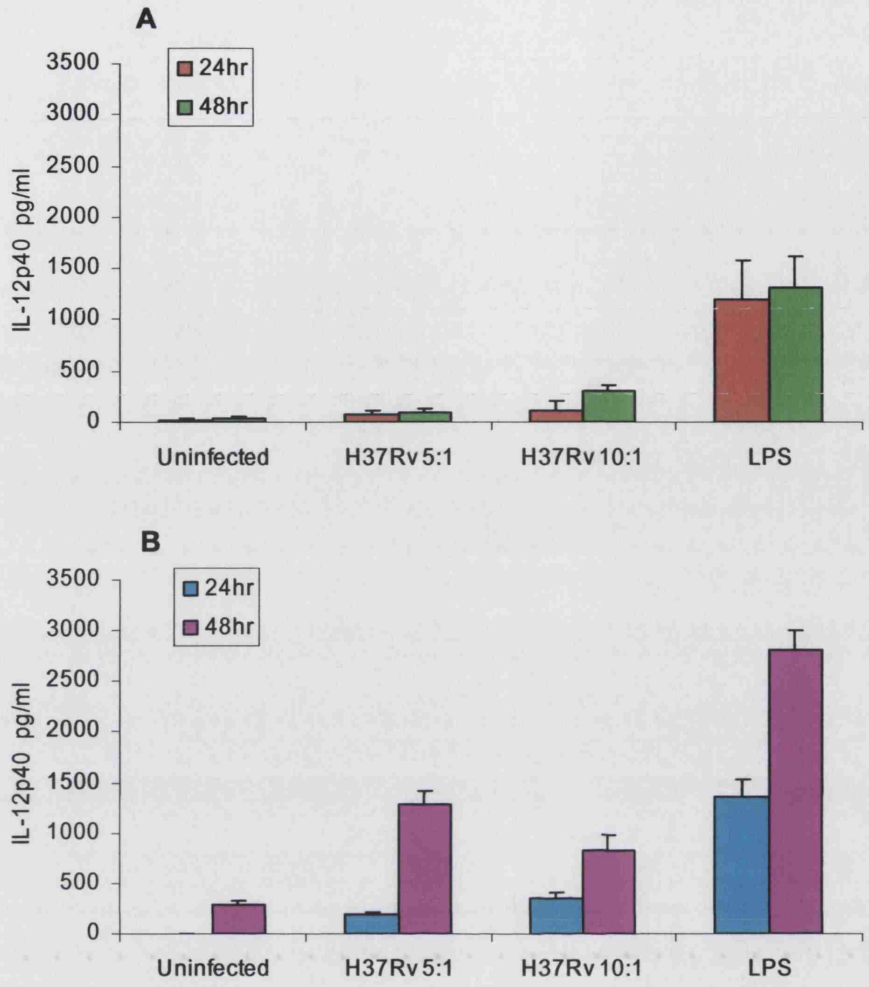

Figure 4.7 IL-12p40 production in response to *M. tuberculosis* infection in unsorted BMDC and sorted CD11c$^+$ BMDC. **(A)** Unsorted BMDC. **(B)** CD11c$^+$ DC. Cells were cultured *in vitro* for 24 or 48 hours and supernatants were collected and analyzed by ELISA for the presence of IL-12p40. Results show the mean +/- SD of 2 independent experiments.

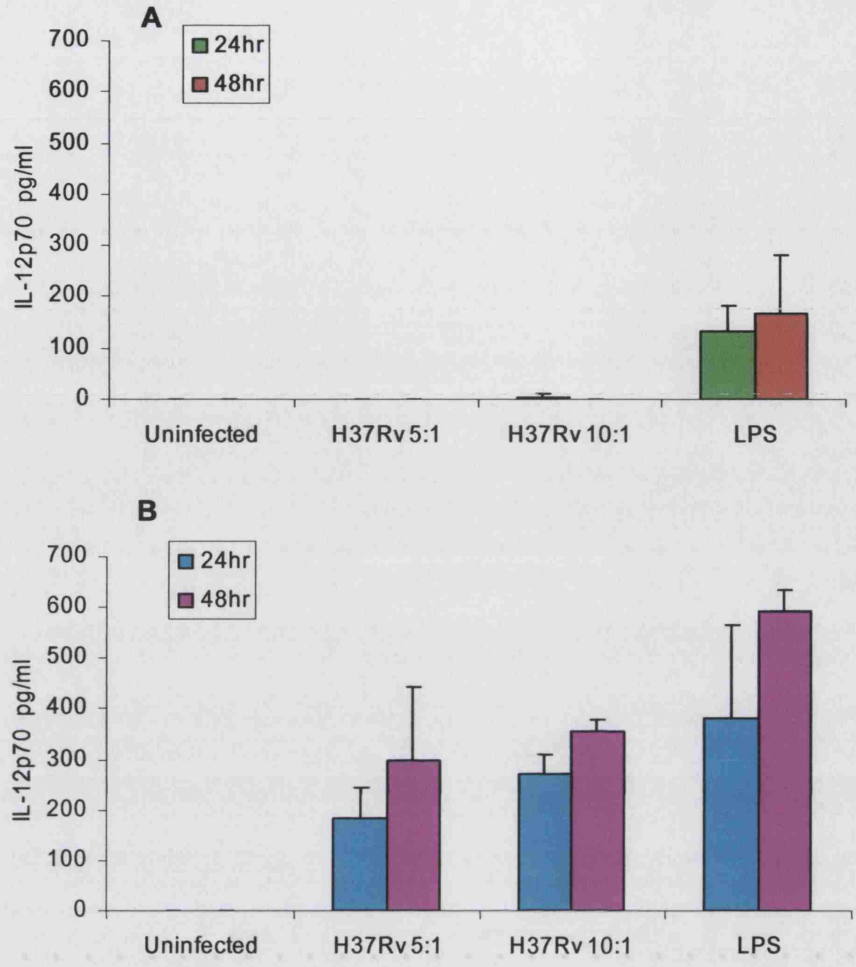

Figure 4.8 IL-12p70 production in response to *M. tuberculosis* infection in unsorted BMDC and sorted CD11c$^+$ BMDC. (A) Unsorted BMDC. **(B)** CD11c$^+$ DC. Cells were cultured *in vitro* for 24 or 48 hours and supernatants were collected and analyzed by ELISA for the presence of IL-12p70. Results show the mean +/- SD of 2 independent experiments.

When conducting these experiments the unsorted BMDC were cultured exactly as described in section 2.2.1, using the supernatant of the Ag8653 cell line as a source of GM-CSF. However, when conducting the experiment using the $CD11c^+$ DC a problem of contamination arose with the Ag8653 cell line, thus the $CD11c^+$ DC were cultured in media containing 10ng/ml recombinant GM-CSF (rGM-CSF; Sigma). It is highly possible that this difference may be responsible for the variance in levels of cytokines, particularly IL-10, produced by the two cell populations. In order to further investigate this problem, the following experiment was conducted.
BMDC were grown as before, and a second batch of cells were grown in identical conditions, except the Ag8653 supernatant was replaced with 10ng/ml rGM-CSF. The cells were infected as before and levels of IL-10 quantified by ELISA. Figure 4.9 indicates that the unsorted BMDC grown in rGM-CSF showed much reduced levels of IL-10, with very low levels being produced by uninfected cells. This factor, therefore did indeed play a key role in the observed difference between unsorted BMDC and $CD11c^+$ DC shown in Figure 4.6, with levels of IL-10 produced by unsorted BMDC grown in rGM-CSF similar to the levels observed in $CD11c^+$ DC. Evidence suggests that DC maturation and function may be altered upon exposure to elevated levels of IL-10 (150). The addition of IL-10 has been demonstrated to block DC maturation in response to LPS stimulation (25). Also, treatment of DC with exogenous IL-10 down-regulates their expression of mRNA for IL-12 and impairs their capacity to induce IFN-γ-producing cells *in vivo* (55). The addition of anti-IL-10 neutralizing antibody or soluble IL-10 receptor to DC cultures can be observed to have opposite effects (45, 79). Thus, IL-10 appears to play a suppressive role on the generation of IL-12-mediated immune responses by DC. (For further discussion of IL-10 and its role in mycobacterial infection see section 1.2.2.4).
Based on these observations, it was thought that the high background levels of IL-10 observed in unsorted BMDC (Figure 4.6) may have been responsible for

suppressing the IL-12 response of these cells (Figure 4.8). Thus, the levels of IL-12p70 were also measured in unsorted BMDC grown in different conditions (Figure 4.10).

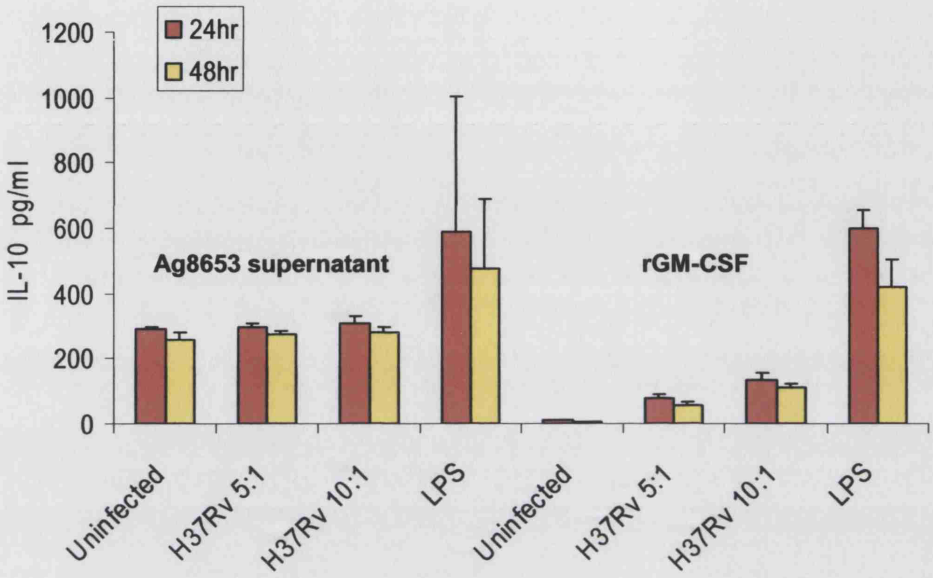

Figure 4.9 IL-10 production in response to *M. tuberculosis* infection in unsorted BMDC grown in Ag8653 supernatant or rGM-CSF. BMDC were cultured in media containing either 10% supernatant of the Ag8653 cell line or 10ng/ml rGM-CSF. Supernatants were collected at 24hr and 48hr and analyzed by ELISA for the presence of IL-10. Results are shown as mean +/- SD of 2 independent experiments.

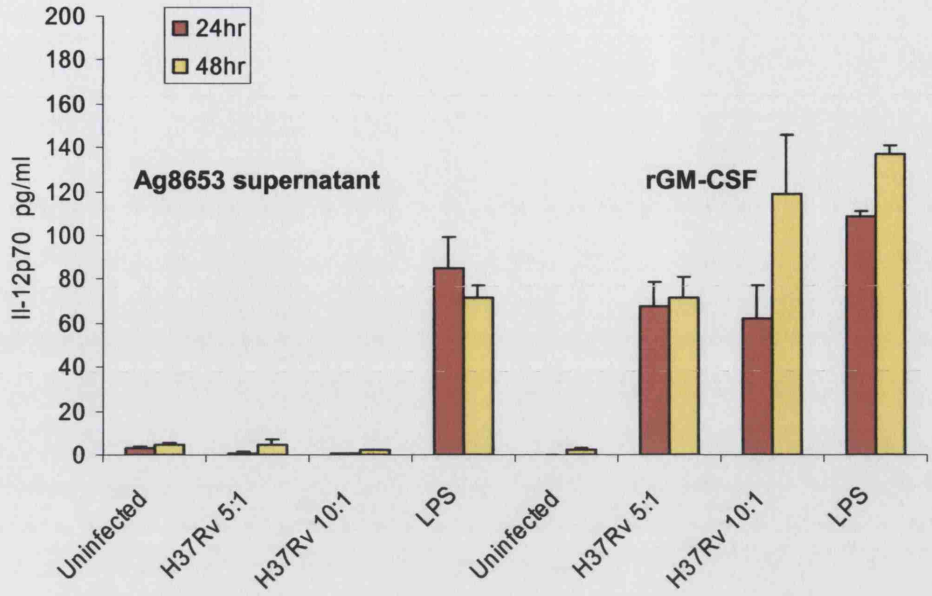

Figure 4.10 IL-12p70 production in response to *M. tuberculosis* infection in unsorted BMDC grown in Ag8653 supernatant or rGM-CSF. BMDC were cultured in media containing either 10% supernatant of the Ag8653 cell line or 10ng/ml rGM-CSF. Supernatants were collected at 24hr and 48hr and analyzed by ELISA for the presence of IL-12p70. Results are shown as mean +/- SD of 2 independent experiments.

The results (Figure 4.10) demonstrate that growth of unsorted BMDC in rGM-CSF resulted in the production of IL-12p70 in response to *M. tuberculosis* infection. Thus, it is likely that the high background levels of IL-10 observed when DC were grown in Ag8653 supernatant (Figure 4.6) were responsible for suppressing the IL-12p70 response to infection by this cell population. However, it should be noted that although unsorted BMDC grown in rGM-CSF produced IL-12p70 in response to infection, the levels observed were not as high as those produced by $CD11c^+$ DC (Figure 4.8).

Chapter 5

Cross-priming *in vivo*

5.1 Introduction

In recent years there has been considerable interest in developing DC-based vaccines for immune prophylaxis or immunotherapy against tumors (30) or infectious agents such as *Chlamydia* and *Toxoplasma* (20, 163). The ability of antigen-pulsed DC to prime protective immune responses in a weakly immunogenic syngeneic tumor model system (30) has emphasised the potential of this approach. Studies using murine models of experimental TB have demonstrated a role for both $CD4^+$ and $CD8^+$ T cell responses (155, 206). The reconstitution of athymic mice with either $CD4^+$ or $CD8^+$ T cells, or a combination of both, revealed that both subsets are able to confer similar levels of protection, and that this protection was only conferred by T cells capable of IFN-γ production (221). This would suggest that the primary function of $CD8^+$ T cells is production of IFN-γ early during infection. Thus, immunization strategies which stimulate both $CD4^+$ and $CD8^+$ T cells would appear to be required for effective protection against *M. tuberculosis* and antigen-pulsed DC have previously been shown to be effective at inducing these two T cell populations (99).

A number of studies have indicated that DC can efficiently phagocytose apoptotic cells and cross-present viral or tumor antigens to $CD8^+$ T cells (1, 2). This pathway corresponds to the *in vivo* phenomenon of cross-priming. The trafficking of exogenous antigen by DC in this cross-presentation pathway is poorly understood, but presentation to T cells via this pathway is particularly efficient (122).

The experiments described in this chapter sought to investigate whether mycobacteria-infected DC could prime a protective response *in vivo* and whether this protection occurred via cross-priming. The ability of mycobacteria-infected DC to stimulate naïve $CD4^+$ and $CD8^+$ T cells *in vivo* was also studied.

5.2 Protective immunity conferred *in vivo* by *M. tuberculosis*-infected DC

The previous chapter revealed that *M. tuberculosis* infection of murine precursor BMDC was sufficient to induce their activation. Experiments were conducted to determine whether these *M. tuberculosis*-infected DC could prime a protective response to subsequent infection with viable *M. tuberculosis*. BMDC were infected *in vitro* with H37Rv and subsequently irradiated at lethal dose. C57Bl/6 mice were injected with either 3 identical IP injections of BMDC alone, *M. tuberculosis* H37Rv-infected irradiated BMDC or irradiated H37Rv, or received a single dose of BCG. Mice were challenged with viable H37Rv and six weeks later counts of viable bacteria were performed on the lungs. The experimental procedure is described in section 2.7 and illustrated in Figure 2.1. The results are shown in Figure 5.1. Naïve mice, those receiving BMDC alone and those receiving irradiated *M. tuberculosis* H37Rv had similar numbers of viable *M. tuberculosis* following challenge infection. Protection was observed in the BCG group; interestingly mice receiving H37Rv-infected and irradiated BMDC showed a protective response better than that achieved with BCG.

As the DC were irradiated prior to injection it is unlikely that these cells were directly presenting mycobacterial antigen *in vivo*. It is more likely that donor cells were taken up by recipient antigen-presenting cells and cross-presented to T cells. We sought to investigate this by repeating the protection experiments using allogeneic DC infected with an auxotrophic mutant of *M. tuberculosis*.

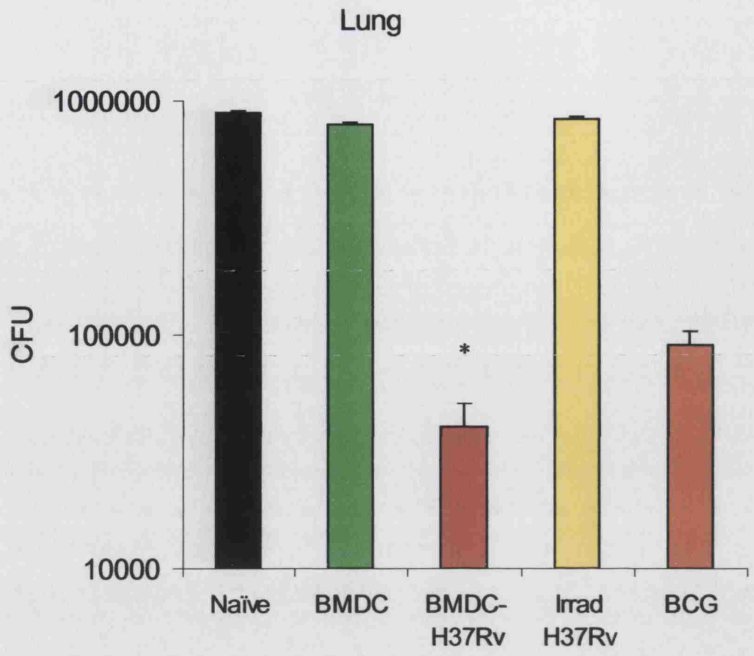

Figure 5.1 Protection induced by *M. tuberculosis* H37Rv-infected irradiated BMDC. C57Bl/6 mice received either uninfected BMDC, H37Rv-infected and irradiated BMDC, irradiated H37Rv or BCG. Mice were then challenged with H37Rv. The number of viable bacteria (mean +/- SEM) was measured in the lungs of 5 mice per group. The BMDC-H37Rv group was significantly different when compared to the naïve group *P<0.01 as measured by the Student's t-test.

5.3 *ΔlysA*: an auxotrophic mutant of *M. tuberculosis*

The *ΔlysA* mutant ($mc^2$3026) is a lysine auxotroph of *M. tuberculosis* (H37Rv) (170). This mutant contains a deletion in the *lysA* gene, encoding *meso*-diaminopimelate decarboxylase, an enzyme involved in catalyzing the final step in lysine biosynthesis. Thus, it is dependent on exogenous supplementation with L-lysine.

This mutant does not replicate in mice, rapid clearance is observed. A similar rate of clearance is observed in SCID (severe combined immunodeficiency) mice, suggesting that clearance was due to an inability to survive in the absence of lysine rather than due to the immune response. A single dose of this *ΔlysA* mutant was shown not to confer protection against subsequent challenge with virulent *M. tuberculosis* (169). The *ΔlysA* mutant was kindly provided by Dr W Jacobs (AEMC, New York) and was used as a model system to further investigate cross-priming. Initially, it was necessary to determine whether the *ΔlysA* mutant could survive and grow within DC and look at the effects of infection on DC viability. Growth experiments were conducted as previously described (section 2.5.1). Cells were infected with *ΔlysA* at an MOI of 1:1 for 6 hours before removing extracellular bacteria and re-culturing the DC in fresh media. L-lysine was included and was maintained at a concentration of 40μg/ml for the duration of the experiment. Intracellular growth of *ΔlysA* was measured in both C57Bl/6 BMDC and the tsDC cell line. This is a temperature-sensitive allogeneic DC cell line and was kindly donated by Dr B Stockinger (NIMR, London). It is transformed with the large T antigen of SV40 and grows at the permissive temperature of 33-35°C, when incubated at 39°C is it induced to mature (236).

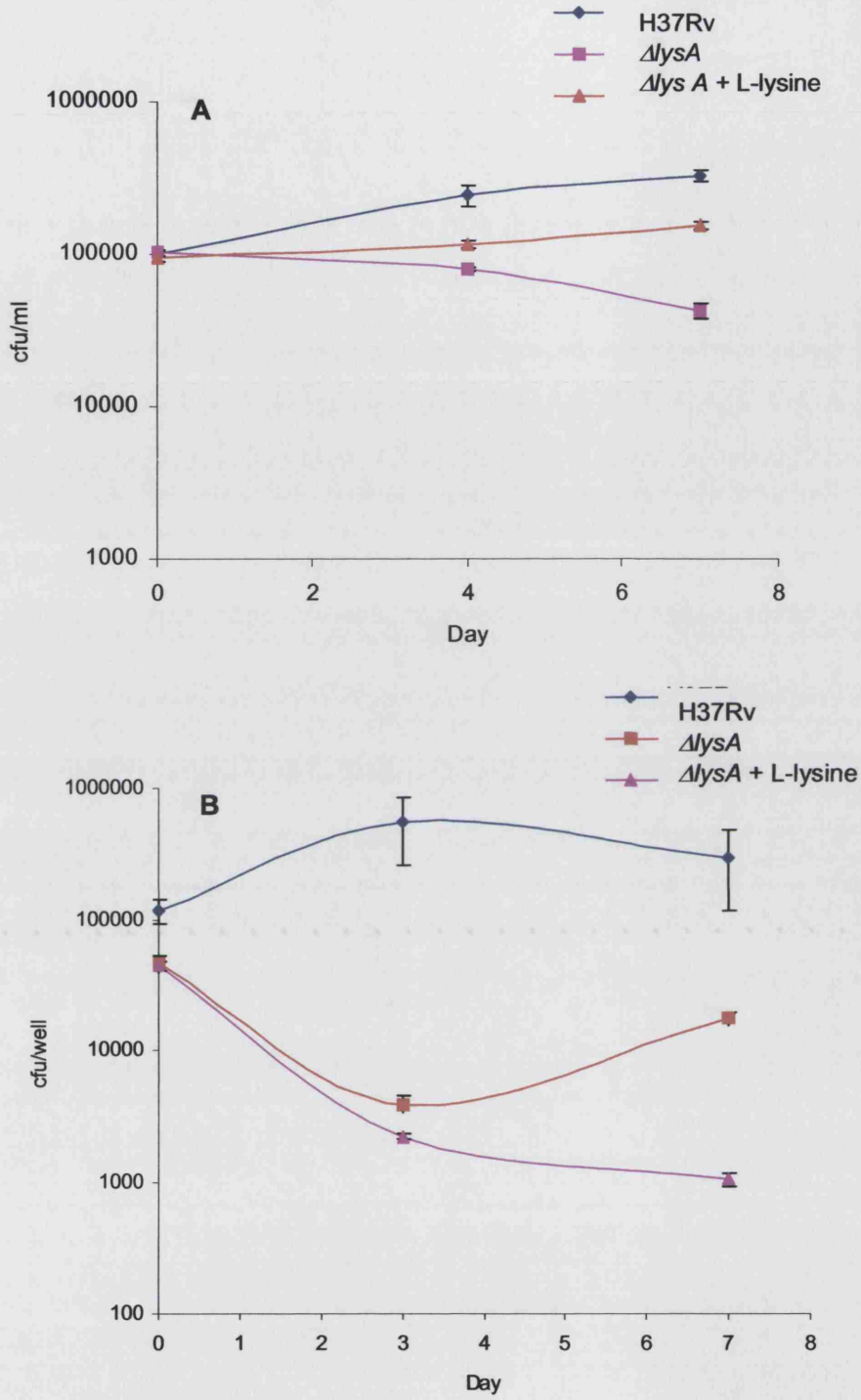

Figure 5.2 Growth of *M. tuberculosis ΔlysA* mutant in DC. (A) C57Bl/6 BMDC. **(B)** tsDC. L-lysine maintained at 40μg/ml where present. Results show mean CFU +/- SE of triplicate wells and are representative of 3 independent experiments.

Figure 5.2 reveals that in the absence of exogenous supplementation with L-lysine the *ΔlysA* mutant fails to sustain viability within DC. When L-lysine was present the mutant was able to survive and replicate within BMDC in a manner similar to H37Rv. In the tsDC cell line, the mutant did not seem to grow as efficiently as observed in BMDC. This is consistent with previous results from our laboratory, indicating that these cells do not support the growth of wild type *M. tuberculosis* as well as do primary BMDC (220). This may be due to the fact that this is a transformed cell line that is continuously growing.

Figure 5.3 shows the percentage of cells infected with *ΔlysA* at an MOI of 5:1 or 10:1 for 6 hours, as determined by acid-fast staining. Also shown is the percentage of viable DC at 48 hours post-infection as determined by Trypan blue exclusion.

Figure 5.4 shows DC viability in response to *M. tuberculosis ΔlysA* infection as measured by cell death detection ELISA assay (see section 2.5.3).

As was demonstrated with *M. tuberculosis* H37Rv in section 3.6, the number of cells infected with the *ΔlysA* mutant could be increased by increasing the ratio of infection, however this was associated with a substantial reduction in cell viability. This was true for both BMDC and the tsDC cell line.

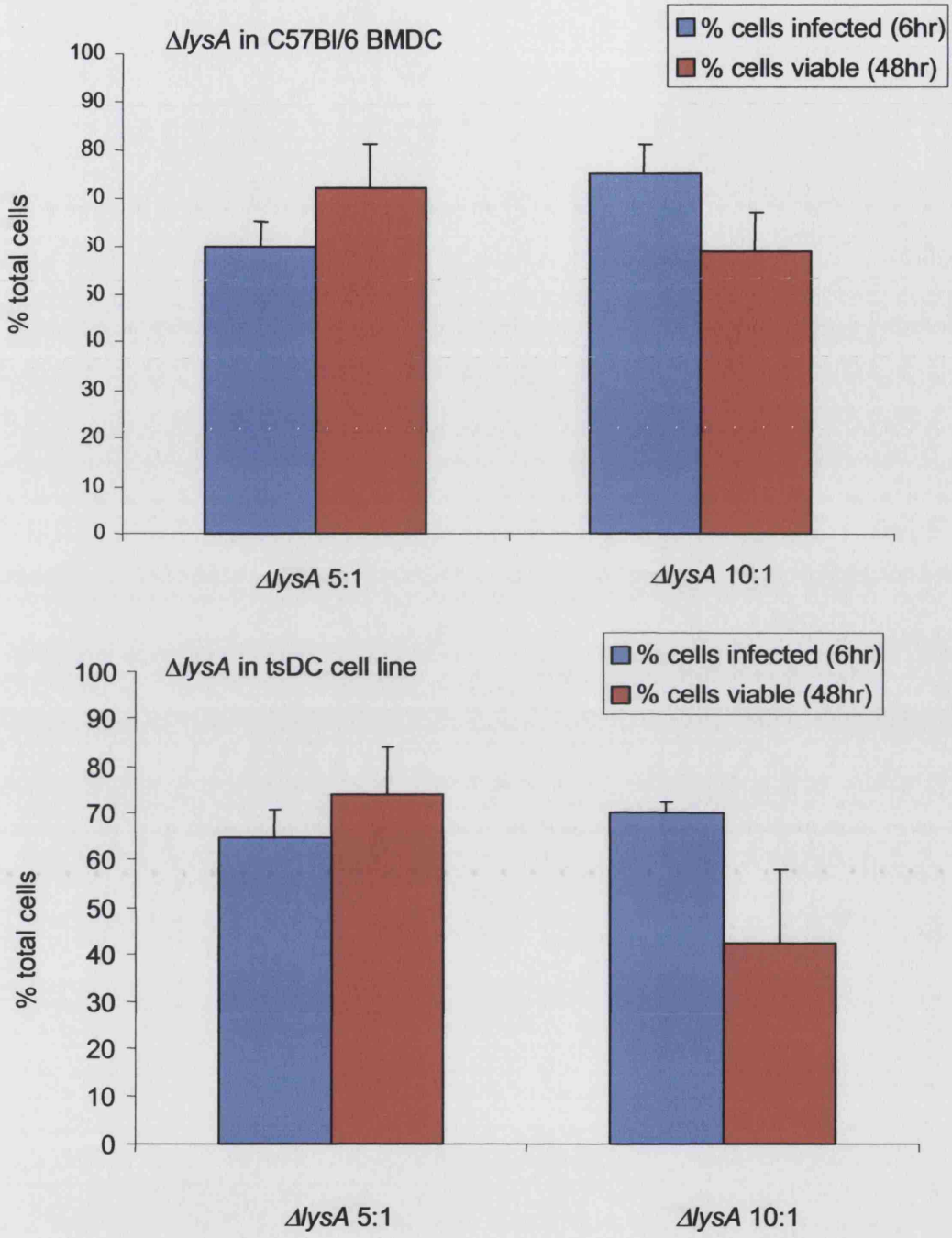

Figure 5.3 Infection of DC with *M. tuberculosis ΔlysA* mutant.

Blue bars show the percentage of infected cells with *ΔlysA* mutant at MOI of 10:1 after 6 hours as measured by acid-fast staining. Red bars show the percentage of viable cells after 48hr culture, as measured by Trypan blue exclusion. L-lysine was maintained at 40μg/ml in all groups. Results show mean +/- SD of triplicate cultures and are representative of 3 independent experiments.

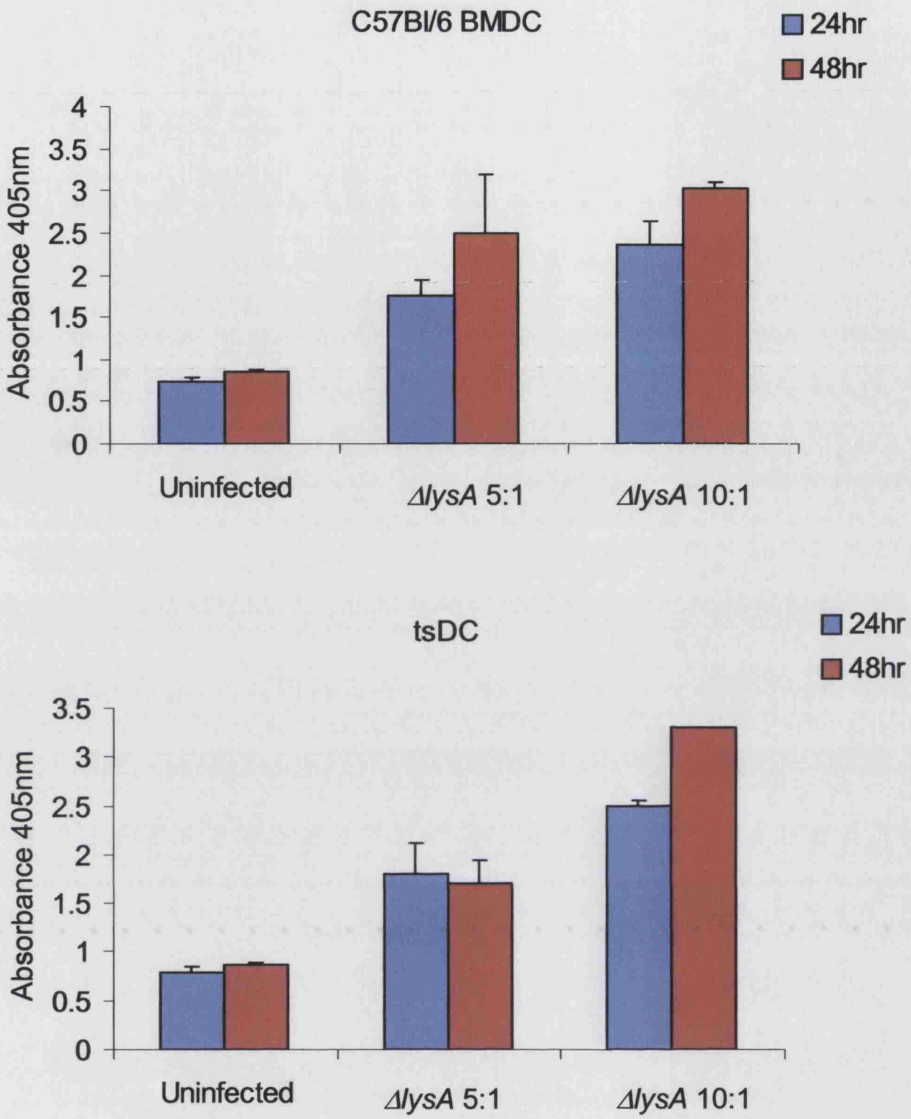

Figure 5.4 Viability of DC infected with *M. tuberculosis* ΔlysA mutant. Viability as measured by cell death detection ELISA. L-lysine was maintained at 40μg/ml in all infected groups. Results show mean +/- SD of triplicate wells and are representative of 2 independent experiments.

5.4 Protection *in vivo* conferred by *M. tuberculosis ΔlysA* -infected DC

The *M. tuberculosis ΔlysA* auxotrophic mutant was used as a model system to further investigate cross-priming *in vivo*. The rationale for this approach derived from the fact that the auxotrophic mutant does not survive for sufficient time in the infected host to induce protective immune responses, due to the inability to survive without exogenous lysine supplementation. By first allowing the mutant to grow *in vitro* within DC in the presence of L-lysine, more efficient antigen processing is promoted. These infected DC can then be administered to mice, since the mutant will be cleared as it is unable to replicate *in vivo* after the supplement is withdrawn. In these experiments, the tsDC cell line was used as a source of allogeneic DC. These cells are from a CBA background (236), thus would be unable to directly present antigens to T cells in vaccinated C57Bl/6 mice as they are of a different haplotype. C57Bl/6 BMDC or tsDC were infected *in vitro* with *M. tuberculosis ΔlysA* at an MOI of 10:1 for 48 hours in the presence of 40μg/ml L-lysine. As shown in section 5.3, this infection protocol results in a substantial reduction in DC viability; and this effect may promote cross-priming. The cells were extensively washed prior to injection to remove free bacteria. C57Bl/6 mice received 3 identical IP injections of C57Bl/6 BMDC alone, *ΔlysA*-infected C57Bl/6 BMDC, tsDC alone, *ΔlysA*-infected tsDC, *ΔlysA* alone or a single dose of BCG. Mice were challenged with virulent *M. tuberculosis* H37Rv and 6 weeks post-challenge the numbers of viable bacteria were determined in spleen and lungs. Results are shown in Figure 5.5.

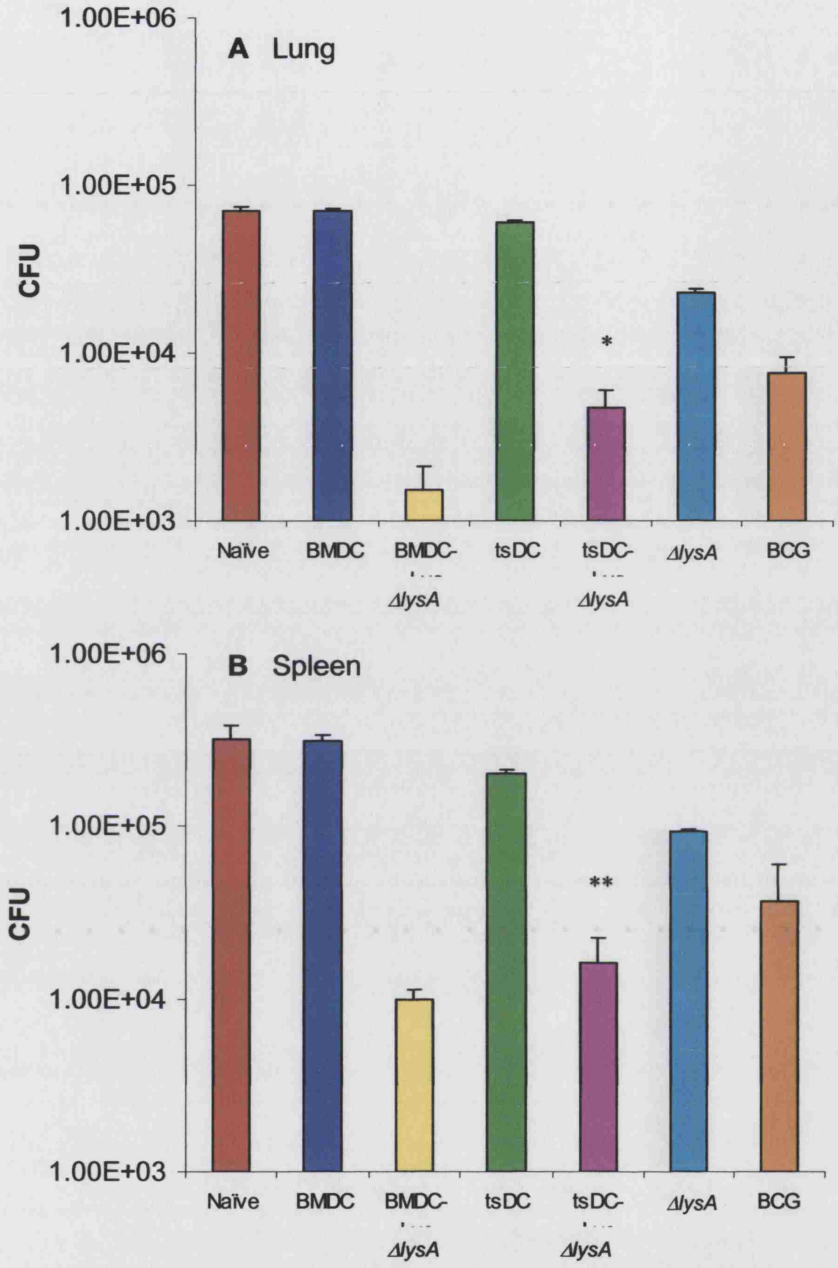

Figure 5.5 Protective immunity induced *by M. tuberculosis ΔlysA*-infected DC. C57Bl/6 mice received either uninfected BMDC, *ΔlysA*-infected BMDC, uninfected tsDC, *ΔlysA*-infected tsDC, *ΔlysA* or a single dose of BCG. At 6 weeks post-injection mice were challenged with *M. tuberculosis* H37Rv. The number of viable bacteria (mean +/- SE) was measured in the lungs **(A)** and the spleens **(B)** of 5 mice per group. the *ΔlysA*-infected tsDC vaccinated group was significantly protected when compared to the naïve or tsDC control groups * $P<0.05$ ** $P<0.002$ as measured by Student's t-test.

These results confirmed that DC infected with this auxotrophic mutant of *M. tuberculosis* induce significant protection in the murine experimental model of *M. tuberculosis* infection, and demonstrate that significant protection can be induced across an allogeneic barrier suggesting effective cross-presentation of antigen from donor infected cells by recipient APC.

5.5 Cross-priming of mycobacteria-specific T cell responses

The previous section revealed that *M. tuberculosis ΔlysA*-infected DC induced protective responses *in vivo* and suggested that effective cross-presentation of antigen has occurred *in vivo*. The following experiments were designed to determine if this protective response was associated with specific $CD4^+$ and $CD8^+$ T cell responses. The vaccination experiment described in section 5.4 was repeated, however rather than challenging the mice, spleens were harvested and $CD4^+$ and $CD8^+$ T cells purified by magnetic cell sorting. The $CD4^+$ or $CD8^+$ T cells were then cultured *in vitro* with irradiated C57Bl/6 splenocytes (to serve as antigen-presenting cells) in the presence or absence of purified protein derivative (PPD; a preparation of mycobacterial peptide antigens). Concavalin A (ConA; a T cell mitogen) was included as a positive control. After 24 and 72 hours culture supernatants were collected for quantification of IL-2 or IFN-γ respectively, by ELISA. After 72 hours cells were intracellularly stained for IFN-γ. The experimental procedure is described in section 2.8 and illustrated in Figure 2.2.

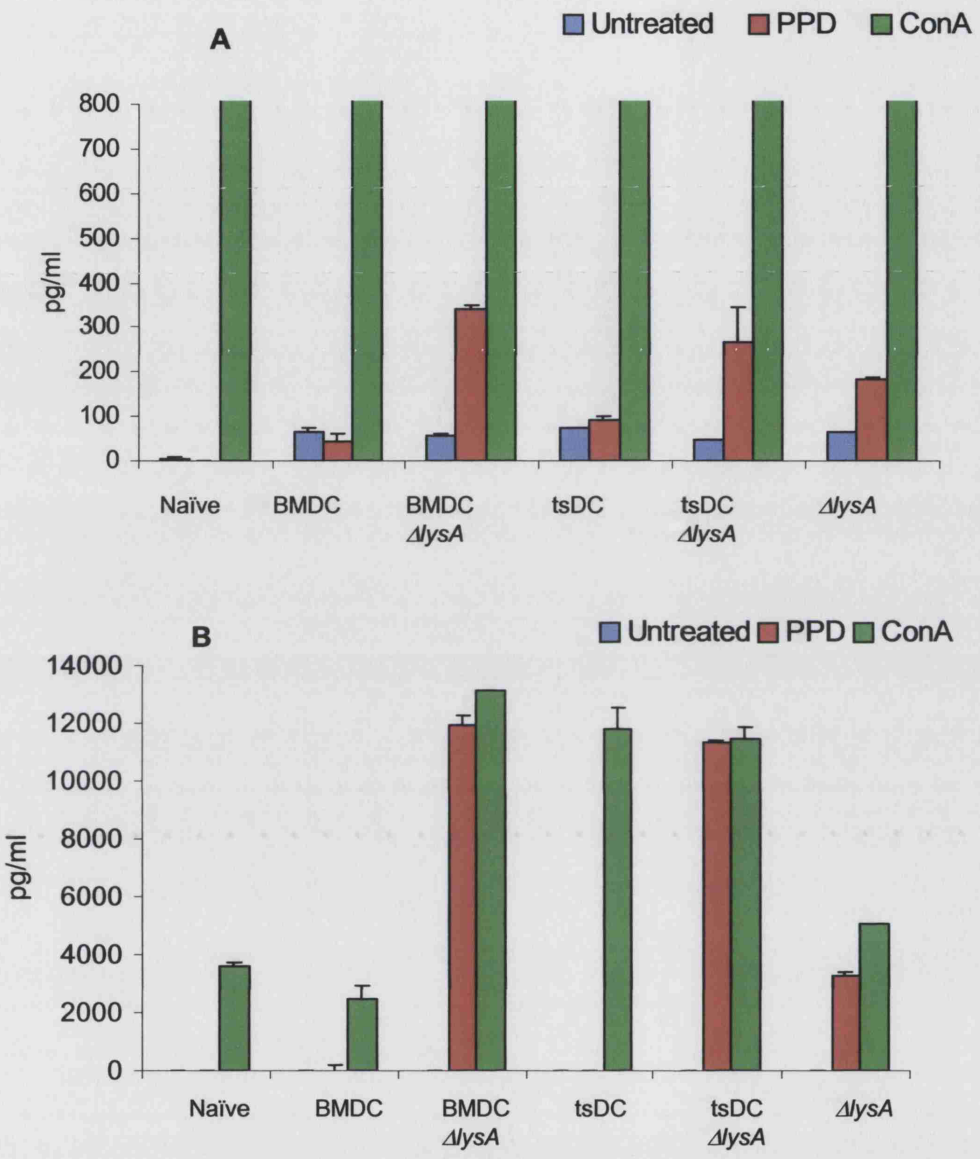

Figure 5.6 IL-2 and IFN-γ production by $CD4^+$ T cells from vaccinated mice. (A) IL-2 measured at 24 hours. All ConA samples were over 3000pg/ml. **(B)** IFN-γ measured at 72 hours. Cells were cultured alone, or in the presence of PPD or ConA as described in section 2.8. Results show mean +/- SD of triplicate wells and are representative of 3 independent experiments.

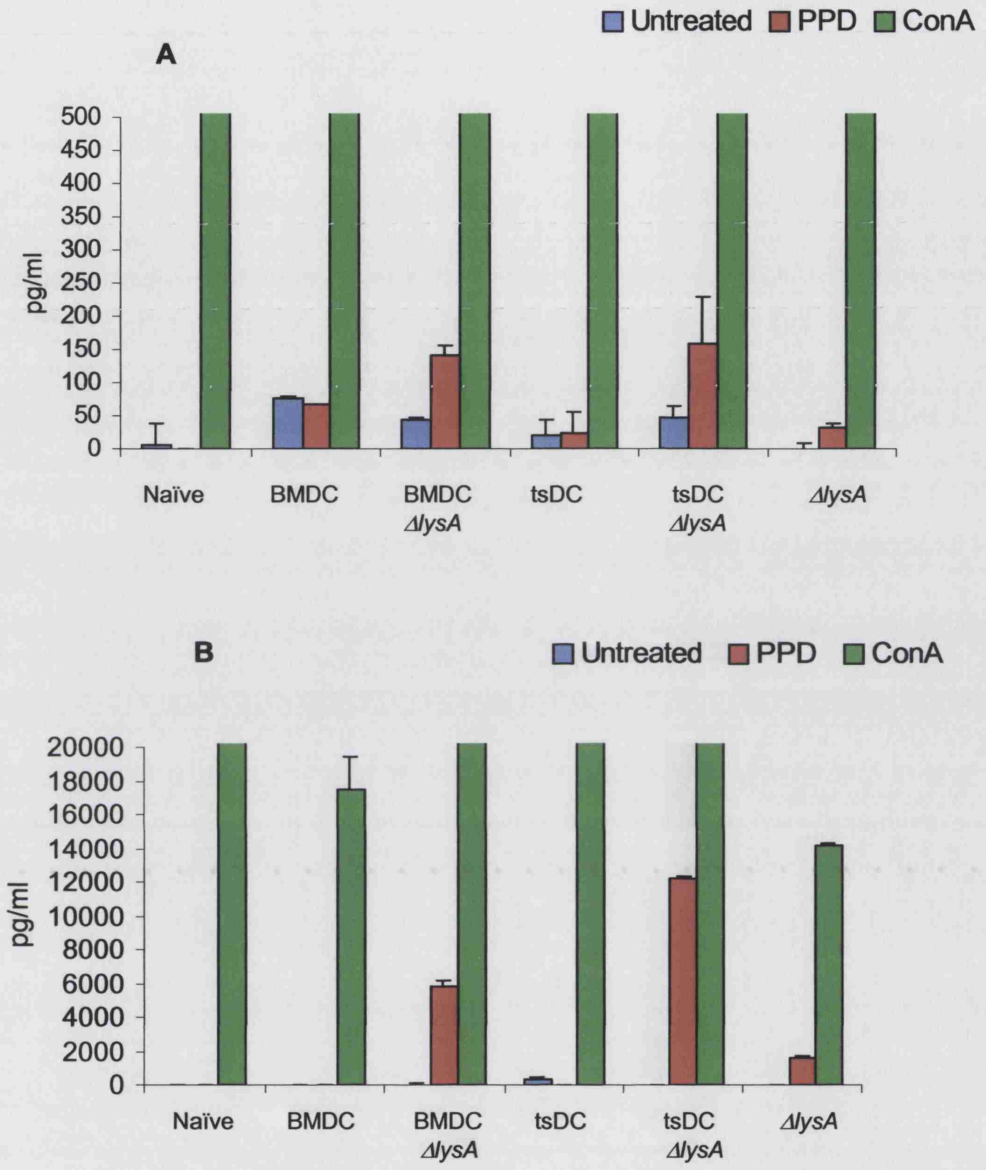

Figure 5.7 IL-2 and IFN-γ production by $CD8^+$ T cells from vaccinated mice. (A) IL-2 measured at 24 hours. All ConA samples were over 3000pg/ml. **(B)** IFN-γ measured at 72 hours. Cells were cultured alone, or in the presence of PPD or ConA as described in section 2.8. Results show mean +/- SD of triplicate wells and are representative of 3 independent experiments.

	%of total cells positive for IFN-γ	
	$CD4^+$ cells	**$CD8^+$ cells**
Naïve	0.6	2.3
ΔlysA	4.1	9.6
BMDC	1.4	3.8
***ΔlysA*-infected BMDC**	7.1	18.4
tsDC	1.5	4.6
***ΔlysA*-infected tsDC**	13.2	25.7

Table 5.1 Intracellular production of IFN-γ by $CD4^+$ and $CD8^+$ cells from vaccinated mice in response to PPD stimulation. $CD4^+$ or $CD8^+$ cells from naïve, *M. tuberculosis ΔlysA*, BMDC, *ΔlysA*-infected BMDC, tsDC or *ΔlysA*-infected tsDC vaccinated mice were cultured *in vitro* in the presence of PPD for 72 hours and were then stimulated and stained as described in section 2.8.4.

As can be seen in Figure 5.6 $CD4^+$ T cells from mice receiving *ΔlysA*-infected DC produced both IL-2 and IFN-γ in response to PPD re-stimulation. No production of IFN-γ and only very low levels of IL-2 were observed in the absence of PPD, indicating that the response was specific for mycobacteria. Table 5.1 demonstrates that in the spleens of mice receiving *ΔlysA*-infected DC there is effective induction of IFN-γ-producing $CD4^+$ T cells as compared to naïve mice or those receiving DC alone. Figure 5.7 shows that $CD8^+$ cells from mice receiving *ΔlysA*-infected DC also produce increased levels of IL-2 and IFN-γ in response to PPD, and this correlated well with the results shown in Table 5.1, where elevated levels of IFN-γ-producing $CD8^+$ T cells were detected. Overall, Table 5.1 would suggest that a higher proportion of IFN-γ-producing cells are of the $CD8^+$ T cell subset.

In both the $CD4^+$ and $CD8^+$ groups there appears to be a cytokine response in the mice receiving *M. tuberculosis ΔlysA* alone. This may be due to the fact that it is

difficult to control the numbers of viable bacteria received by the groups injected with *ΔlysA*-infected DC. Although each mouse received an identical number of infected cells, the number of viable bacteria present in each dose was unknown at time of injection. Thus, it is likely that those mice receiving bacteria alone were given a much higher dose of viable *ΔlysA* than those receiving infected DC.
These results reveal that the protective response demonstrated in these experiments is due to cross-presentation of mycobacterial antigen to both $CD4^+$ and $CD8^+$ T cells.

5.6 Depletion of $CD4^+$ or $CD8^+$ T cells in mice vaccinated with *M. tuberculosis ΔlysA*-infected DC

The previous section revealed that the protective response observed *in vivo* correlated with specific $CD4^+$ and $CD8^+$ T cell responses. In order to determine the relative contribution of these T cell subsets in the protective response, the protection experiment described in section 5.4 was repeated, including groups in which $CD4^+$ or $CD8^+$ T cells were antibody depleted.

5.6.1 Depletion of $CD4^+$ or $CD8^+$ T cells *in vivo*.

The monoclonal antibodies YTS 191.1, specific for the L3T4 determinant expressed on $CD4^+$ T cells and YTS169.4, specific for the Lyt-2 determinant on $CD8^+$ T cells were kindly provided by Dr B Stockinger (NIMR, London) and are described in (38). These monoclonal antibodies are of rat origin and are of the IgG2b subtype. This subclass of antibody is capable of destroying cells expressing their target antigen *in*

vivo, probably via activating the complement system or antibody-dependent cellular cytotoxicity, or both. Prior to including these antibodies in our vaccination experiments, it was necessary to test the levels of depletion attained *in vivo*. Naïve C57Bl/6 mice received 0.1mg of either YTS 191.1 or YTS 169.4 via the IP route. At 5 days and 21 days post-injection the lungs and spleens were harvested and flow cytometry conducted to ascertain the proportion of $CD4^+$ or $CD8^+$ cells present. Results are illustrated in Figures 5.8 and 5.9 and are summarized in Table 5.2.

	Day 5		**Day 21**	
	% cells $CD4^+$	**% cells $CD8^+$**	**% cells $CD4^+$**	**% cells $CD8^+$**
Spleen:				
Untreated	30.2	14.5	27.4	13.5
YTS 191.1	18.3	15.0	20.5	13.9
YTS 169.4	35.9	1.1	26.0	7.4
Lung:				
Untreated	11.9	9.3	25.4	6.7
YTS 191.1	0	10.4	15.6	5.2
YTS 169.4	11.3	0	18	3.2

Table 5.2 Summary of antibody depletion in naïve C57Bl/6 mice.

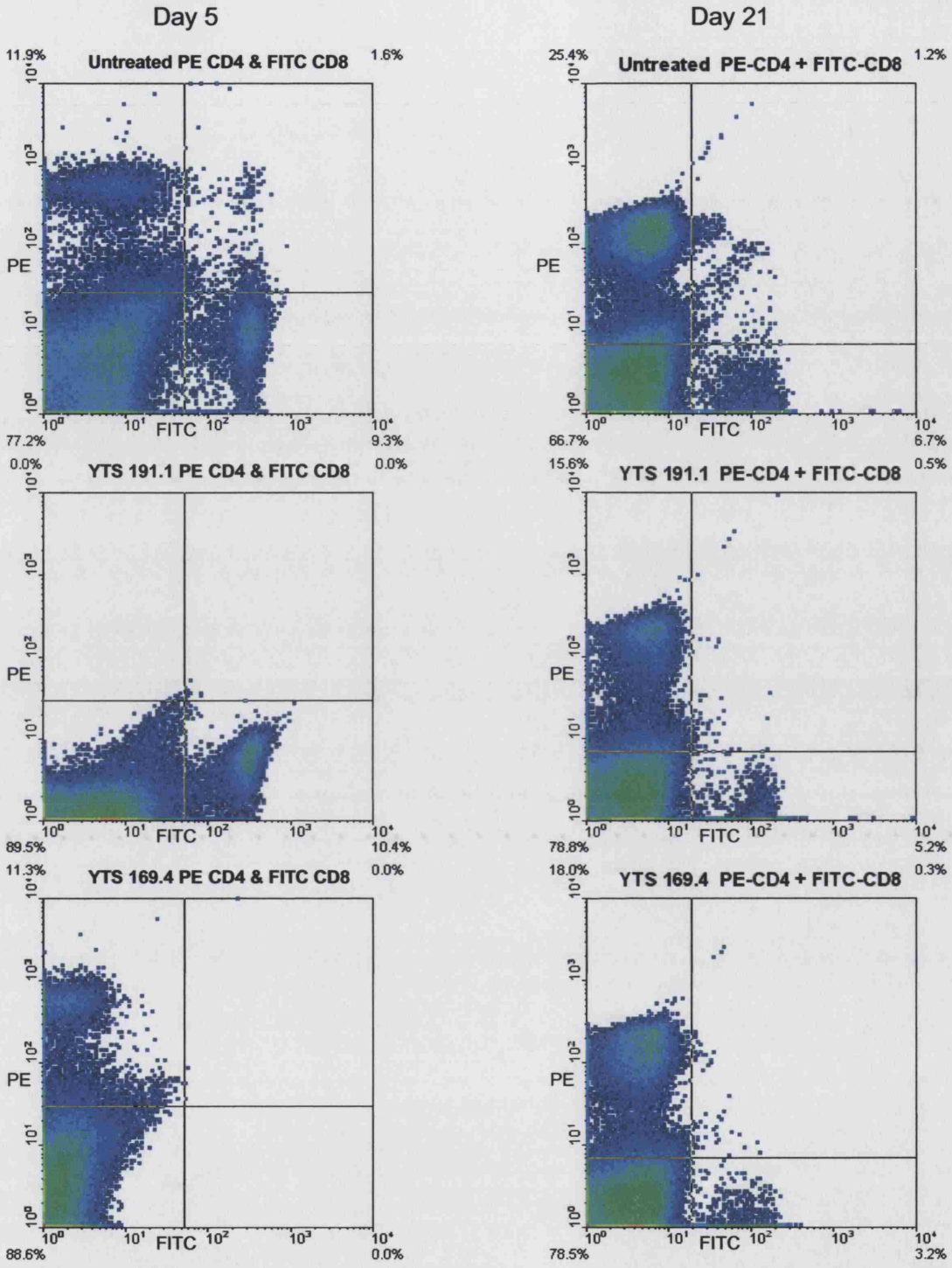

Figure 5.8 Depletion of CD4$^+$ and CD8$^+$ T cells in lungs of C57Bl/6 mice. Mice were injected with anti-CD4 (YTS 191.1) or anti-CD8 (YTS 169.4) and flow cytometry carried out on days 5 and 21 post-injection.

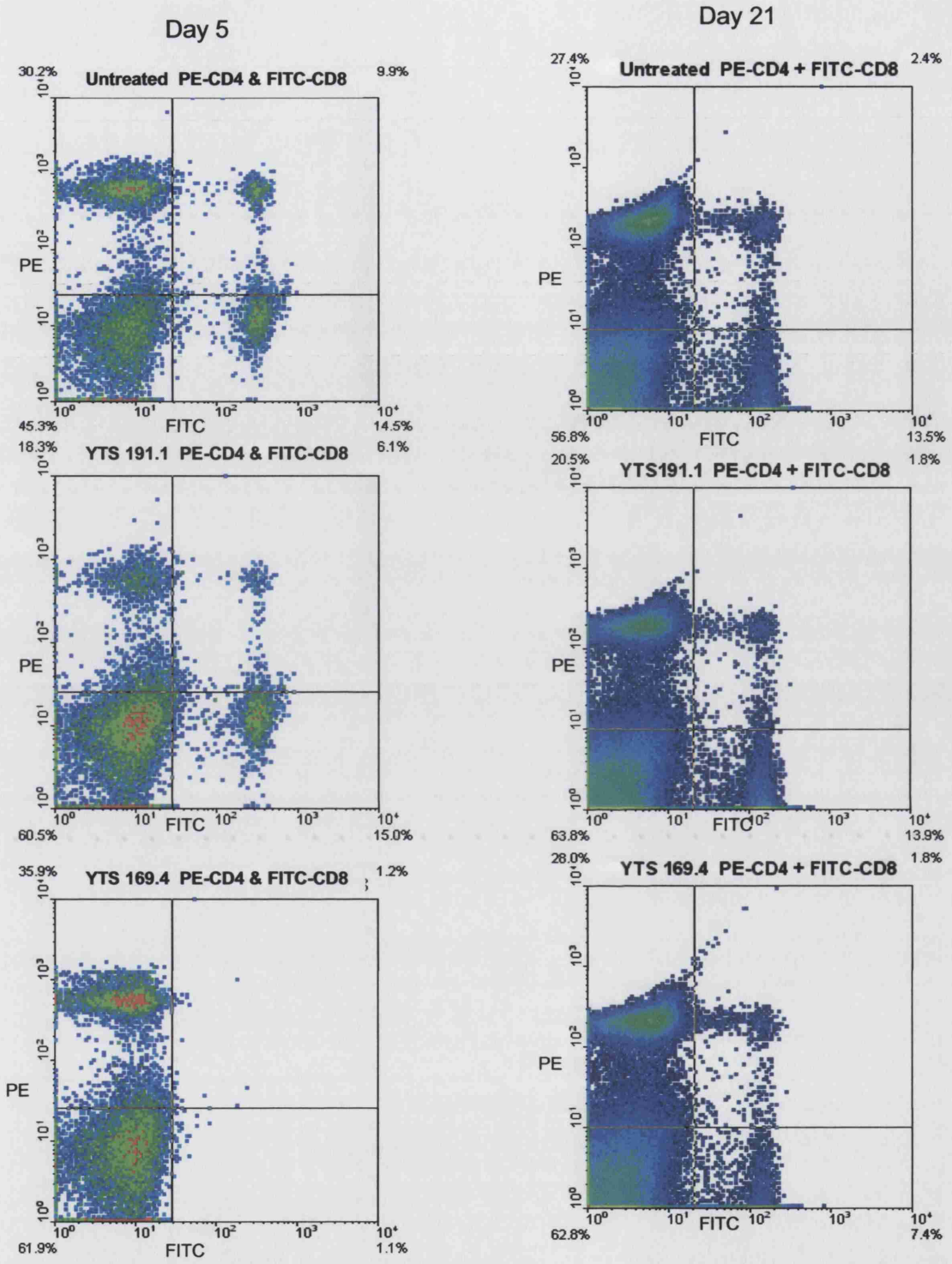

Figure 5.9 Depletion of $CD4^+$ and $CD8^+$ T cells in spleens of C57Bl/6 mice. Mice injected with anti-CD4 (YTS 191.1) or anti-CD8 (YTS 169.4) and flow cytometry carried out on days 5 and 21 post-injection.

As can be seen from these results at day 5 post-treatment, a significant proportion of $CD4^+$ and $CD8^+$ T cells were depleted in the lungs and spleens, with $CD8^+$ T cell depletion being almost complete. By day 21 post-treatment the cell numbers were beginning to recover but are not completely reconstituted. Thus, it was decided to include these antibodies in our vaccination model to study if depletion of these cell populations had any detrimental effect on the protective response.

5.6.2 Depletion of $CD4^+$ or $CD8^+$ T cells in mice vaccinated with *M. tuberculosis ΔlysA*-infected DC.

The protection experiment described in section 5.4 was repeated. On this occasion three groups of mice received *M. tuberculosis ΔlysA*-infected tsDC; one group was untreated, one group received anti-CD4 antibody (YTS 191.1) and one group received anti-CD8 antibody (YTS 169.4), as described in section 2. As before, all mice were challenged with virulent *M. tuberculosis* H37Rv 6 weeks following the final injection of cells. In addition, as we demonstrated that *M. tuberculosis ΔlysA*-infected DC contained a large proportion of dead cells (as shown in Figure 5.3 and Figure 5.4), it was decided to include an additional group in this experiment, to investigate if increasing the proportion of dead cells could further promote increased levels of protection. Mice in this additional group received tsDC which had been infected with *M. tuberculosis ΔlysA* and were then placed at -80°C to induce necrosis. The cells injected into these mice were 100% non-viable as measured by Trypan blue exclusion. Results of this experiment are illustrated in Figure 5.10.

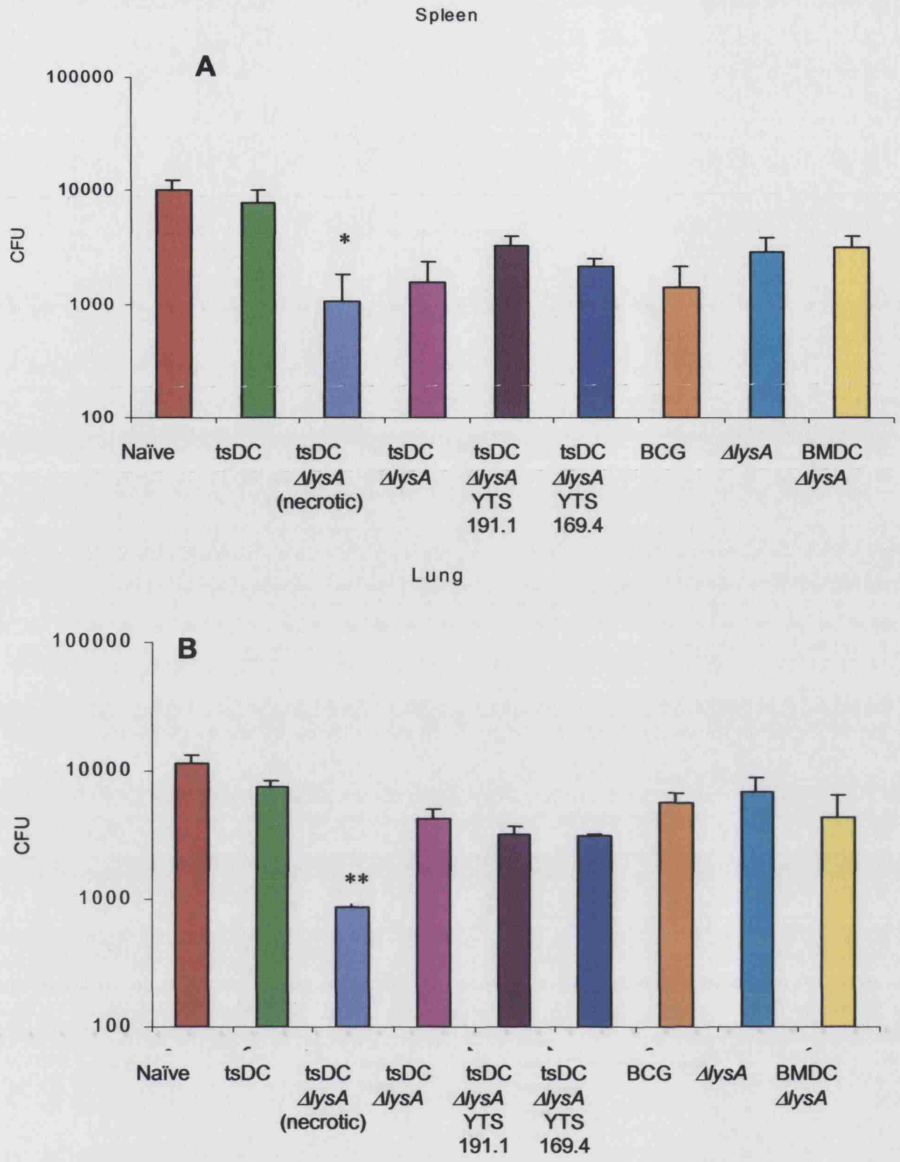

Figure 5.10 Depletion of CD4$^+$ or CD8$^+$ T cells in mice vaccinated with *M. tuberculosis* ΔlysA-infected DC. C57Bl/6 mice received either *ΔlysA*-infected BMDC, uninfected tsDC, *ΔlysA*-infected tsDC, *ΔlysA*-infected necrotic tsDC, *ΔlysA* alone or a single dose of BCG. Two of the *ΔlysA*-tsDC groups were treated with YTS191.1 or YTS169.4 as described. Mice were challenged with *M. tuberculosis* H37Rv. The number of viable bacteria (mean +/- SE) was measured in the spleens **(A)** and the lungs **(B)** of 5 mice per group. *P<0.05 and **P<0.002 compared to *ΔlysA*-tsDC group, as measured by Student's t-test.

Results demonstrated that depletion of $CD4^+$ or $CD8^+$ T cells had no effect on the levels of protection in comparison to *ΔlysA*-infected tsDC. The second dose of depletion antibodies was administered three weeks prior to challenge infection and as shown in section 5.6.1, by 21 days post-treatment cell numbers were beginning to recover. Thus, the lack of effect observed in these groups may be attributable to the fact that the $CD4^+$ and $CD8^+$ T cell numbers had recovered sufficiently by the time the mice were challenged. Due to the constraints of time it was not possible to repeat this experiment with a shorter interval between the doses of depletion antibody.

Interestingly, the mice receiving necrotic *M. tuberculosis ΔlysA*-infected tsDC showed significantly improved protection over those receiving *ΔlysA*-infected tsDC.

5.7 Protective immunity induced by *M. tuberculosis ΔlysA*-infected DC in a guinea pig model of TB

Having demonstrated effective priming of a protective response across an allogeneic barrier (section 5.4), it was decided to test whether a protective response could also be induced using this vaccination protocol in a xenogeneic model. This experiment was conducted in a guinea pig model of experimental TB infection, in collaboration with Ann Williams (CAMR, Porton Down). Animals received three identical injections of $2.5x10^6$ cells at 4 week intervals. After 4 weeks animals were challenged via the aerosol route with virulent *M. tuberculosis* H37Rv and 6 weeks later viable counts were performed on the spleens and lungs. Results are illustrated in Figure 5.11.

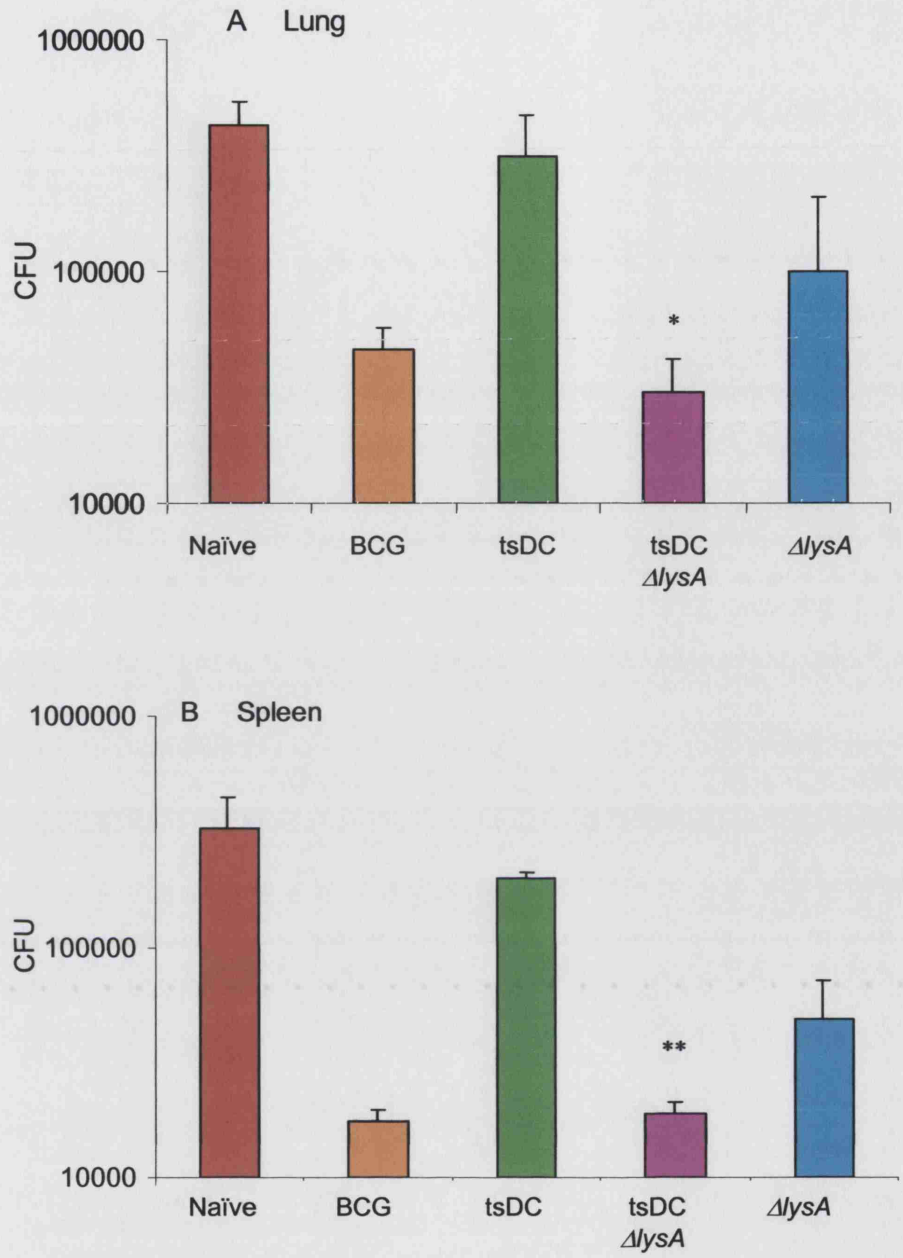

Figure 5.11 Protective immunity induced by *M. tuberculosis* ΔlysA-infected tsDC in guinea pigs. Groups of guinea pigs received 3 identical injections of either tsDC, *ΔlysA*-infected tsDC, *ΔlysA* alone or a single dose of BCG. 4 weeks post-injection animals were challenged with *M. tuberculosis* H37Rv. The number of viable bacteria (mean +/- SE) was measured in the lungs (A) and the spleens (B) of 6 animals per group. The *ΔlysA*-infected tsDC vaccinated group was significantly protected when compared to the naïve group * $P<0.05$ ** $P<0.001$ as measured by Student's t-test.

This experiment revealed a significant level of protection conferred by *M. tuberculosis ΔlysA*-infected tsDC, similar to that achieved by BCG. The ability to transfer protection across a xenogeneic barrier suggests that cross-presentation of mycobacterial antigens also occurred in this model.

Chapter 6

Discussion

DC are recognized to be the most important cells involved in the priming of naïve T cells, therefore the response of these cells to mycobacterial infection remains an area of intense study (89, 93, 220). The overall aim of this work was to investigate the interaction of murine primary precursor DC with *M. tuberculosis* and to examine the ability of mycobacterially-infected DC to cross-prime a protective response *in vivo*.

The initial experiments described in Chapter 3, were aimed at determining whether *M. tuberculosis* could survive and replicate within DC and also to observe if these cells could control the intracellular growth of the pathogen. DC have been identified in the airway epithelium and lung parenchyma (98) and have been previously shown to be readily infected by *M. tuberculosis* (93), thus it is likely that they play a crucial role in the initiation of protective immune responses.

Murine precursor BMDC were found to phagocytose *M. tuberculosis* and to permit the intracellular growth of the bacteria in a manner similar to that observed in BMMϕ, as assessed by acid-fast staining and by lysis of the cells and determining the number of viable bacteria in the original cultures. It is well established that activated murine Mϕ are capable of inhibiting the intracellular growth of *M. tuberculosis* by producing increased levels of RNI (32), our results show that precursor DC activated by IFN-γ are also capable of controlling intracellular growth, as observed by a reduction in bacterial numbers recovered over the course of the experiments. As DC have previously been shown to have the capacity to produce NO (17), we speculated that this could be the mechanism by which they inhibited intracellular growth of *M. tuberculosis*. Measurement of NO levels in supernatants collected from infected cells confirmed that IFN-γ activated DC produce significant levels of NO in response to *M. tuberculosis* infection. The results of experiments using the competitive inhibitor of iNOS, L-NAME, strongly suggested that NO production is at

least one mechanism by which activated DC can control intracellular bacterial growth.

It was suspected that the growth inhibition and NO production observed in these experiments were due to the presence of contaminating Mϕ within the BMDC cultures. To address this issue $CD11c^+$ sorted DC were used to repeat these experiments and these results confirmed that $CD11c^+$ sorted DC were able to express the mycobactericidal effects observed. However, it is also possible that uninfected DC within the culture were activated to exert bactericidal effector mechanisms. We observed that increasing the MOI resulted in a greater percentage of infected cells, however this was also associated with a loss of DC viability, thus we could not conduct growth experiments as the cells did not survive for sufficient time.

Other studies investigating the ability of pathogens to survive within DC have shown that *Salmonella* (143) and *Leishmania* (149) appear to survive within DC, however *Chlamydia* are killed by a phagosome-lysosome fusion pathway (163). The constraints of time did not permit further investigation into the mechanisms used by DC to control intracellular bacterial growth. However, it may be of interest to investigate the possible production of ROI by DC and their effects on bacterial growth.

Immature DC are specialized in antigen capture and following antigen encounter are induced to activate into a mature antigen presenting cell, migrating to lymphoid tissues to present antigen to specific T cells and producing cytokines which influence T cell differentiation. The experiments described in Chapter 4 have investigated some of these changes induced in unsorted BMDC and $CD11c^+$ DC in response to *M. tuberculosis* infection.

The results confirmed that infection of DC by *M. tuberculosis* induced the up-regulation of costimulatory molecules (CD40, CD80 and CD86) and antigen

presenting molecules (MHC class II) on the cell surface, consistent with a change to an activated phenotype. No difference was observed between the expression of these cell surface molecules on unsorted BMDC and $CD11c^+$ DC. The up-regulation of these molecules on the cell surface is consistent with an increase in antigen presenting capacity. It has previously been suggested that Mϕ infected with mycobacteria have diminished capacity to present antigen, particularly in the context of MHC class II to $CD4^+$ T cells, and this has been attributed to either reduced expression of MHC class II or inhibition of antigen processing (159, 160). Our results demonstrate an up-regulation in the expression of MHC class II on DC in response to *M. tuberculosis* infection. Taken together with other studies demonstrating that DC possess unique pathways for antigen processing and the intracellular trafficking of peptide-MHC class II complexes, (110) it could be speculated that the reduced antigen presenting capacity observed in Mϕ may not be found in mycobacterially-infected DC. This area of the effects of *M. tuberculosis* infection of DC on their antigen presenting capacity requires further investigation, however it was out with the scope of this work.

The activation of DC in response to *M. tuberculosis* infection was also associated with the elaboration of pro-inflammatory cytokines in response. In particular, the production of bioactive IL-12 was of interest. IL-12 is essential in the control of *M. tuberculosis* infection as it drives the differentiation of T cells towards a Th1 type response, with the production of IFN-γ, which is associated with protective immunity against mycobacteria. In general, $CD11c^+$ DC produced higher levels of cytokines in response to infection than did the unsorted BMDC population. The finding that culture of cells using the Ag8653 supernatant as a source of GM-CSF resulted in high background levels of IL-10 could have wide ranging practical implications. This cell line is widely used as a source of GM-CSF and the IL-10 induced may skew responses away from Th1 towards a Th2 type response. IL-10 production by the

unsorted BMDC population was completely abrogated when the cells were grown in rGM-CSF and in keeping with the immunosuppressive effects of IL-10, cells grown in rGM-CSF showed an improved IL-12 response to infection. However, cells (both unsorted BMDC and $CD11c^+$ DC) grown in rGM-CSF did still show production of IL-10 in response to infection. This may represent a negative feedback mechanism to regulate the inflammatory response and thus prevent immunopathology *in vivo*.

A recent report suggested that *M. tuberculosis* induces only a limited maturation of human DC and may impair DC function (89). This study utilized human DC derived from peripheral blood mononuclear cells (PBMC) rather than murine BMDC. Also, the cells were infected at a relatively low ratio of infection (MOI 3:1) and activation markers were measured only at a single time point (2 days post-infection). It is clear that in this report *M. tuberculosis*-infected cells did indeed show a reduced activation phenotype, when compared to the activation phenotype used as a positive control. However, when compared to uninfected controls the DC could be seen to have undergone some degree of activation.

It should be noted that the experiments undertaken in Chapter 4 were originally conducted including a group with a lower ratio of infection (MOI 1:1). However, this low inocula did not induce significant activation of the DC (neither cell surface marker expression or significant levels of cytokine production). Thus, it would appear that a higher ratio of infection is required to promote DC activation. This may explain why *M. tuberculosis* infection alone did not significantly activate the DC to control intracellular growth of the bacteria in the experiments detailed in Chapter 3, Also, this may have implications for the progression of disease *in vivo*, low infectious dose may result in insufficient DC activation and thus affect the subsequent immune response to the pathogen.

This study has utilized DC generated from bone marrow-derived progenitors, whereas in natural infection the port of entry for TB is most commonly the lung.

Interactions between *M. tuberculosis* and lung DC have been studied, however to a certain extent much remains to be learned. One study observing murine lung DC from BCG-infected mice demonstrated that these cells produced high levels of IL-12p40, however in contrast to our findings, and those of others (16) IL-12p70 was not detected (125). These differences may be attributable to the complex interactions of lung DC with the bacilli *in vivo* as opposed to DC infected *in vitro*.

In summary, the results presented in Chapter 4 were consistent with previous reports that BCG (48) or *M. tuberculosis* (93, 220) infection results in the activation of DC from an antigen capturing to an antigen presenting cell. The efficient priming of T cells requires not only the recognition of antigenic peptides in association with MHC molecules, but also costimulation via the engagement of molecules such as CD80 and CD86 by their ligands on T cells. The observed up-regulation of these molecules on DC following *M. tuberculosis* infection suggests that these cells are induced to become mature antigen-presenting cells, which are likely to play a principle role in the initiation of anti-mycobacterial T cells responses. The elaboration of pro-inflammatory cytokines, particularly IL-12, would promote the generation of a protective Th1 type response. The observed up-regulation of CD40 in response to *M. tuberculosis* infection may also be of importance; in addition to promoting further DC activation upon ligation of CD40L on T cells, CD40 ligation may also influence the nature of the resulting T cell response. It has been suggested that ligation of CD40 during APC-T cell interactions may promote IL-12 production and the generation of a Th1 type protective response.

Chapter 5 describes a series of experiments which sought to investigate if mycobacteria-infected DC could prime a protective immune response *in vivo*, whether this response involved specific $CD4^+$ and $CD8^+$ T cell priming and whether this protective response was effective across allogeneic and xenogeneic barriers.

Initial experiments revealed that *M. tuberculosis*-infected and irradiated syngeneic DC induced significant levels of protection against subsequent challenge with virulent *M. tuberculosis* and that the protection conferred was greater than that achieved by BCG. These cells were lethally irradiated, thus it is unlikely that these cells were directly presenting mycobacterial antigens *in vivo*, a more plausible scenario would involve the donor cells being taken up by recipient APC, which then cross-present mycobacterial antigens to specific T cells in the host. A model utilizing the *ΔlysA* ($mc^2$3026) auxotrophic mutant of *M. tuberculosis* allowed further study of the protective response *in vivo*. This mutant is a lysine auxotroph and its survival is dependent on exogenous supplementation with L-lysine (169, 170). It is rapidly cleared *in vivo* and thus does not survive long enough for a protective immune response to be mounted, due to its inability to survive in the absence of exogenous L-lysine. The results using this mutant showed that *M. tuberculosis ΔlysA* can infect DC and cause a reduction in DC viability in a manner similar to that observed with wild type *M. tuberculosis* H37Rv.

Syngeneic BMDC or allogeneic tsDC which had been infected with *M. tuberculosis ΔlysA in vitro*, were shown to induce a protective response against subsequent challenge with *M. tuberculosis* and importantly the protection achieved was greater than that provided by BCG. The tsDC cell line was generated on a CBA background (236), thus are of a different haplotype to the C57Bl/6 recipients. Therefore these cells could not be directly presenting mycobacterial antigens *in vivo*. This ability to transfer protection across an allogeneic barrier, strongly suggests that the observed protection was achieved by cross-priming in this model. It was also revealed by use of a guinea pig model of TB infection, that immunization with *M. tuberculosis ΔlysA*-infected tsDC could induce protection across a xenogeneic barrier, thus confirming that protection was induced by cross-priming. The guinea pig experiment also indicated that utilizing the aerosol route of infection appeared to have no detrimental

effect on the protection achieved. This was a concern as the experiments conducted in mice used the IV infection route, and we were concerned that aerosol infection may result in a smaller infectious dose, possibly resulting in suboptimal DC activation and affecting the resultant immune response. However, this remains to be confirmed by repeating these experiments in mice using the aerosol route of infection.

The protective response conferred by *M. tuberculosis ΔlysA*-infected DC was shown to be associated with both specific $CD4^+$ and $CD8^+$ T cells, responding to restimulation with mycobacterial antigen in a Th1 manner. Thus, it can be concluded that immunization with DC infected with this *M. tuberculosis* auxotroph resulted in the cross-priming of mycobacteria-specific $CD4^+$ and $CD8^+$ T cells and significant levels of protection against *M. tuberculosis* challenge infection. Antibody depletion of $CD4^+$ or $CD8^+$ T cells had no significant effect on the levels of protection observed in this model. This is in contrast to a study utilizing the same depletion antibodies which demonstrated that selective depletion of either T cell subset enhanced the growth of mycobacteria *in vivo,* indicating that resistance was impaired due to this depletion (152). This study however utilized thymectomised mice and also the antibodies were administered 2-4 days apart and challenged immediately following antibody administration. It is thought that we saw no significant difference in the antibody depleted groups due to the timing of the antibody treatment. As shown in figures 5.8 and 5.9 by day 21 the $CD4^+$ and $CD8^+$ cells were re-populating the tissues, thus by the time our animals were challenged with H37Rv the cell numbers would be recovering from depletion. The constraints of time and a limited supply of the depletion antibodies prevented further investigation of the depletion study.

Mycobacterial infection has previously been shown to induce apoptosis of host cells (193) and the results presented in Chapters 3 and 5 indicate that both *M.*

tuberculosis H37Rv and *ΔlysA* induced a substantial reduction in DC viability, thus a large proportion of the cells administered to mice were dead. Several studies have described the uptake and cross-presentation of antigens derived from dead cells, using a variety of antigen sources, including infectious agents such as influenza (2), *Salmonella* (246) and mycobacteria (193). As a large percentage of the cells injected in our model were non-viable, it was suspected that this promoted cross-presentation of mycobacterial antigens derived from the dead cells. To test this hypothesis, *M. tuberculosis ΔlysA*-infected necrotic tsDC were administered and this vaccination protocol achieved greater levels of protection than previously observed. This may be attributable to several factors; the fact that a greater proportion of dead cells represent a higher dose of antigen, also necrotic, but not apoptotic, cells can activate DC (190). Thus, the administration of infected necrotic cells may provide a source of mycobacterial antigen and a stimulatory signal to activate the host APC *in vivo*, thus resulting in optimal cross-presentation and an enhanced protective response.

A recent report (193) has demonstrated that mycobacterial infection induces apoptosis of host cells and the release of apoptotic vesicles. These vesicles were taken up by uninfected bystander APC and cross-presented to T cells *in vitro*. The inhibition of apoptosis completely abrogated antigen transfer and subsequent T cell activation. These researchers have proposed a new 'detour' pathway for the cross-presentation of antigens from phagosome-contained intracellular pathogens, in which apoptotic infected cells serve as a source of antigen for uptake and presentation by uninfected APC – reviewed in (241). Results presented in Chapter 5 support this hypothesis and demonstrate that cross-priming of both $CD4^+$ and $CD8^+$ T cells *in vivo* by vaccination with DC infected with an *M. tuberculosis* auxotrophic mutant induces a very high level of protective immunity against mycobacterial infection.

It should be noted that the *M. tuberculosis ΔlysA* mutant utilized in our model of DC vaccination would not be suitable as a potential vaccine candidate as it contains only a single mutation (170). Any reversion of this mutation may therefore results in the restoration of virulence. For such safety reasons, auxotrophic mutants considered as potential vaccine candidates should contain multiple separate mutations in order to safeguard their avirulence.

The results in this study have consistently demonstrated levels of protection similar to, or improved upon that achieved with BCG. Most attempts to confer protection greater than that seen with BCG in small rodent models have failed to do so. This approach provides us with an opportunity to investigate further immunological correlates of immunity.

Other groups have used similar DC-based vaccination strategies to induce protection against infectious agents. *M. bovis* BCG-infected DC transferred into the lungs of mice were shown to induce protection against subsequent challenge with virulent *M. tuberculosis* via the aerosol route (48). Similarly, DC pulsed with *Toxoplasma gondii* antigens have been shown to induce protective immunity (20). This study shows for the first time that the induction of a protective response to mycobacteria using *M.* tuberculosis-infected DC is, at least partially, attributable to cross-priming and that the resulting immune response consists of both $CD4^+$ and $CD8^+$ T cells of the Th1 type.

Although the use of cell-based vaccines for TB seems somewhat impractical due to limitations for large-scale prophylactic immunizations, these findings suggest routes by which this approach might be developed for practical application. For example, polynucleotide vaccine constructs encoding pro-apoptotic molecules and mycobacterial antigens could induce enhanced protection. Alternatively, this model for inducing high levels of anti-mycobacterial protective immunity could be combined with other approaches in the future, to give novel prime-boost vaccination strategies.

This model of cross-priming also provides an opportunity to further study the processes of cross-presentation and cross-priming *in vivo*. For example, areas of interest include; whether the route of cell death (apoptosis versus necrosis) is important for the outcome of the immune response; the trafficking and processing of antigen derived from dead cells and the identity of the cross-priming APC *in vivo*. Overall this report has revealed that:

- DC are readily infected with *M. tuberculosis*, and following activation with IFN-γ, these cells can control the intracellular growth of the pathogen via RNI production.
- Infection of DC with *M. tuberculosis* is sufficient to induce their activation.
- Immunization with DC infected with an auxotrophic mutant of *M. tuberculosis* induces a protective immune response involving both $CD4^+$ and $CD8^+$ T cells and that this response is achieved by cross-priming.

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